NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AS A TOOL TO ASSESS PATHOPHYSIOLOGICAL CHANGES IN LIVER DISEASE

by

KONSTANTINOS JOHN DABOS

A Thesis submitted for the degree of

Doctor of Philosophy

Liver Unit

Department of Clinical and Surgical Sciences

University of Edinburgh Medical School

August 2000

To Sophia with love and gratitude

To the memory of my parents

DECLARATION

I hereby declare that this thesis contains an account of my own research work and is based on the results of my own experiments. This thesis is exclusively of my own composition. The data presented in this thesis has not been previously submitted, in whole or in part, for any degree at this or any other university.

Konstantinos J Dabos

August 2000

Acknowledgements

I would like to thank my supervisors Professor Peter C Hayes and Dr. Ian H Sadler for their advice, support and ongoing interest in this study.

I am grateful to Dr. John N Plevris for his invaluable support and advice, for his continuous encouragement and for stimulating discussions throughout the course of this research work. Without his help this project would not have been completed.

My special thanks go to Drs. John A Parkinson and Chandralal M Hewage who have initiated me and guided me into the world of NMR Spectroscopy.

I would like to thank my fellow postgraduate student and collaborator Mr. Leonard Nelson who has helped me with the cell culture experiments.

I gratefully acknowledge the financial support of the Scottish Hospital Endowments and Research Trust to this research work.

I wish to express my gratitude to all past and present colleagues in the Department for stimulating conversations and for collection of blood samples from patients.

Finally, I would like to thank my family for their support. I would particularly like to thank my in-laws for their financial support during the difficult first year.

ABSTRACT

Nuclear magnetic resonance (NMR) spectroscopy has been successfully used in the analysis of biological fluids. Analytical NMR studies of biofluids have been concerned primarily with the collection, assignment and quantitation of NMR spectra and their interpretation in biochemical terms.

^{1}H NMR Spectroscopy has been used over the years to study low molecular weight substances in a variety of biological fluids such as urine, plasma, bile and cerebrospinal fluid in normal controls and in the context of diseases. Many studies have shown the usefulness of the technique in identifying abnormal substances. Few studies have been undertaken to try and quantitate low molecular weight substances in biological fluids.

The aim of this thesis was twofold. First we wanted to validate the use of ^{1}H NMR spectroscopy as a reliable technique to observe and quantitate low molecular weight substances in the context of liver disease. We then wanted to provide some insight into the performance of key metabolic pathways in hepatocytes that are either cultured or failing in the native liver of patients .

We validated our technique by comparing concentrations of low molecular weight substances measured by NMR with results from standard biochemical assays and HPLC. We used primary porcine hepatocytes and studied the effect of different culture conditions and modalities on the metabolism of those cells by looking at the supernatant of the cell cultures. We have shown in this thesis that culture conditions and culture media are factors that greatly influence the performance of key metabolic pathways of cultured hepatocytes and this in turn, influences the life span of cell in culture.

We then investigated the changes in key metabolites in the plasma from patients suffering from chronic liver failure and its complication chronic hepatic encephalopathy and from patients with acute liver failure and compared those concentrations with controls. Key metabolic pathways were impaired in patients with chronic liver failure compared to controls. Further impairment of branch chain amino acids and ketone bodies metabolism was noted in chronic hepatic encephalopathy. In acute liver failure gluconeogenesis was greatly impaired along with ketogensis at the early stages of the disease.

In conclusion NMR spectroscopy is capable of providing accurate estimations of concentrations of low molecular weight substances in biofluids and thus can give us an insight in the pathogenesis of cell dysfunction in liver disease.

Table of Contents

List of Figures

List of Tables

Table 9.1 Individual characteristics of patients with cirrhosis and cirrhosis and encephalopathy. 279

AIM OF THE THESIS

The aim of this thesis was twofold. First we wanted to validate the use of ^{1}H NMR spectroscopy as a reliable technique to observe and quantitate low molecular weight substances in the context of liver disease. We then wanted to provide some insight into the performance of key metabolic pathways in hepatocytes that are either cultured or failing in the native liver of patients.

We have used primary porcine hepatocytes and studied the effect of different culture conditions and modalities on the metabolism of those cells by looking at the supernatant of the cell cultures. We have studied different culture and preservation conditions and have investigated the activity of primary porcine hepatocytes, in an effort to provide accurate data on the function of those hepatocytes in culture.

We have studied biochemical pathways of liver cells in patients suffering from acute liver failure from different aetiologies and we have studied key intermediate metabolites in an effort to link the observed clinical course of the patients with the underlying biochemical abnormalities.

We have also studied biochemical pathways of liver cells in patients suffering from chronic liver failure and chronic hepatic encephalopathy and we have studied differences between key intermediate metabolites in an effort to provide an explanation for the observed symptoms in chronic liver failure and encephalopathy. Interesting data were accumulated and they are looked into in detail in the experimental chapters of this thesis.

CHAPTER 1

INTRODUCTION TO NMR SPECTROSCOPY AND LIVER DISEASES

1.1 NMR Spectroscopy of biological fluids.

Biofluids have been regarded for a long time as important tools to study the biochemical status of a living organism. It is well known that the composition of these fluids is dependent on the function of the cells that are responsible for their manufacture. In the case of blood, abnormalities in its composition can reflect the function of many internal organs. Nuclear magnetic resonance (NMR) spectroscopy has been used successfully in the analysis of biological fluids (Nicholson et al, 1989). Analytical NMR studies of biofluids have been concerned primarily with the collection, assignment and quantitation of NMR spectra and their interpretation in biochemical terms.

Even the advent of powerful spectrometers has not made possible so far the complete assignment of the ^{1}H NMR spectrum of most biofluids. This is due to the enormous complexity of the fluid itself, although some well regulated fluids like seminal fluid, with relatively few metabolites have been almost completely assigned at 750MHz. Others like urine are difficult to interpret even at 1GHz due to the enormous variability of their composition (Lindon et al, 1999). Things are made more difficult with the presence of many metabolites in concentrations close to the limit of detection. All biological fluids have their own characteristic physicochemical properties and that restricts the type of NMR experiments that can be performed on

them and the information that can be gathered from each one. It is generally accepted though that NMR studies of biological fluids should be performed at the highest available field to obtain maximal dispersion and sensitivity and to minimise the noise (Nicholson et al, 1995).

Two types of noise may be evident in the ^{1}H NMR spectra of biological fluids. The most common is the instrument noise which is an inherent property of the apparatus. There is also the chemical noise which is related to the sample itself. (Lindon et al, 1999). Chemical noise is the result of overlapping of signals that are low in abundance in the matrix and close to the detection limits of the spectrometer. Chemical noise is the factor limiting the information recoverable from the spectra of most biofluids. Increasing the frequency can allow recovery of information that was in the chemical noise at lower frequencies.

Information in a spectrum can be classed as patent or latent (Nicholson et al, 1995). Patent information can be recovered from a single pulse experiment in a quantitative fashion. Latent information in an NMR spectrum measured at a particular field is not readily extractable from a single pulse experiment. The biochemical data contained therein can be extracted by carefully selecting the appropriate multiple pulse sequences. If we increase the field strength then information latent in the chemical noise can become patent. Arguably, NMR spectroscopy of biological fluids has become a field of exciting developments and one that has gained a lot from the recent technological advances in spectroscopic methods and instrumentation.

1.2 History of NMR Spectroscopy

NMR Spectroscopy is one of the most important tools for obtaining detailed information on chemical systems at a molecular level. Nuclear magnetic resonance is a nuclear phenomenon related to the magnetic properties of the nuclei. It is based on the ability of some atomic nuclei oriented by a magnetic field (0.70 -20.0 T) to absorb electromagnetic radiation at particular frequencies in the radiofrequency part of the spectrum.

It was first described as a phenomenon in 1946 by two groups. Purcell's group was working at Stanford University (Purcell et al, 1946) and Bloch's group was working at the MIT (Bloch et al, 1946). Purcell and Bloch shared the Nobel Prize for Chemistry in 1952 for this discovery. Useful chemical applications of NMR became possible only after the discovery of the chemical shift effect in 1949 (Dickinson, 1950).

The first high resolution NMR spectra were recorded by the continuous wave (CW) technique. In this method the spectra is recorded either by continuously altering the transmitter frequency whilst the applied magnetic field ($\mathbf{B_0}$) is held constant or more commonly, vice-versa. The first commercial CW proton NMR spectrometer was produced in 1953. As a consequence to this achievement the use of NMR as a chemical tool has increased , but the CW method was only suitable for the recording of spectra of sensitive and high abundance nuclei such as ^{1}H, ^{31}P and ^{19}F. The introduction of the Fourier Transform (FT) method in 1966 by Ernst and Anderson, (Ernst et al, 1966) revolutionised the field. It allowed for the accurate observation of low abundance nuclei such as ^{13}C and ^{15}N. It also allowed for a significant gain in sensitivity. In 1971 Jeener introduced the idea of two dimensional

FT NMR (Jeener, 1971). Around the same time the first commercial FT NMR spectrometers were made available by the Bruker company.

Since then there has been an enormous progress in the instrumentation and methods of NMR Spectroscopy. The construction of high field spectrometers, the introduction of multi-dimensional NMR techniques and the huge advance in computer technology have made some enormous advances in NMR spectroscopy possible.

In the first publication on NMR spectroscopy of biofluids in 1979, Ohsaka et al. reported that 100 MHz ^{1}H NMR spectra of blood serum from patients with malignant tumours showed high lactate levels (Ohsaka et al, 1979). Since then NMR spectroscopy of biofluids has progressed a great deal and the advent of new powerful instruments has allowed for this technique to be used for the investigation and measurement of subtle biochemical processes in complex fluids and cell extracts.

1.3 Theory of NMR Spectroscopy.

NMR Spectroscopy is based upon the magnetic properties of the atomic nucleus. All atomic nuclei possess nuclear spin angular momentum (P). The spin angular momentum P is in units h/2p where h is Planck's constant. According to the quantum theory the angular momentum is quantized:

$$|P| = h\sqrt{I(I+1)}\ /2\pi \qquad (1)$$

The associated quantum number (I) takes a value of 0, $^1/_2$, 1, $1^1/_2$, 2 up to 6. For nuclei with even mass numbers and even charge, I=0 and these nuclei e.g. ^{12}C, ^{16}O, show no magnetic behaviour. Nuclei that have even mass numbers and odd charge e.g. ^{2}H, ^{14}N, have integral values of I. Nuclei with odd mass numbers have half-integral values of spin. Such nuclei are ^{1}H, ^{13}C, ^{15}N and ^{19}F.

The spin angular momentum P has associated with it a magnetic moment (μ). These two vector quantities are proportional to each other and the proportionality constant ? is called the gyromagnetic or magnetogyric ratio.

$$\mu = \gamma P \qquad (2)$$

By combining (1) and (2), the magnetic moment μ also quantized can be obtained.

$$|\mu| = \gamma h\ \sqrt{I(I+1)}\ /2\pi \qquad (3)$$

In a magnetic field ($\mathbf{B}_0$) the orientation (?) of the magnetic moment (μ) to the field direction (z-axis) is also quantized and depends on I. The orientation to the x- and y- axes is not quantized and is undefined. When a magnetic field ($\mathbf{B}_0$) is applied, the angular momentum component P along the direction of the field, conventionally defined as the z-direction, takes values equal to a multiple of m_I h/2p

where m_I is the magnetic quantum number which characterises the corresponding stationary states of the nucleus.

$$|P_z| = m_I \, h/2\pi \qquad (4)$$

As the energy of the nucleus is quantized then m_I can take any of the values m_I = I, I-1, I-2 .... -I giving 2I+1 possible orientations (Figure 1.1) (Purcell et al, 1946). From equations (2) and (4) the component of the magnetic moment along the applied field direction z (μ_z) can be obtained.

$$|\mu_Z| = m_I \gamma \, h/2\pi \qquad (5)$$

The magnetic moment μ never lines exactly with the field direction but is at an angle

$$\{\cos\theta = m \sqrt{I(I+1)}\}$$

and it behaves as if it precesses about the field direction with an angular velocity (?) which is given by the equation ? = -? $\mathbf{B_0}$ (Figure 1.2) where ? is referred to as the Larmor frequency (Bloch et al, 1946).

The energy (E) of a magnetic dipole in an applied field ($\mathbf{B_0}$) is the dot product of two vectors the magnetic moment ($\boldsymbol{\mu}$) and the applied field ($\mathbf{B}$).

$$E = - \boldsymbol{\mu} \mathbf{B} = - \mu \cos\theta \, \mathbf{B_0} = - \mu_z \mathbf{B_0} \qquad (6)$$

$$E = - m_I \, \gamma \, h \, \mathbf{B_0} / 2\pi \qquad (7)$$

Thus the energy difference between the two adjacent energy levels can be given as

$$\Delta E = \gamma \, h \, \mathbf{B_0} / 2\pi \qquad (8)$$

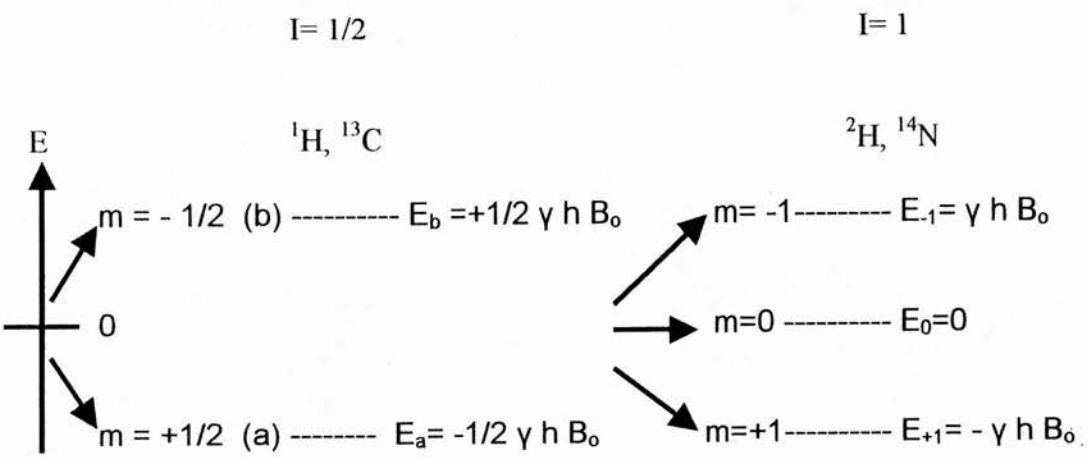


Figure 1.1 Energy level schemes for nuclei I= ½ and I=1

The selection rule for NMR transitions between orientations is $\Delta m = \pm 1$. Transition between the two adjacent energy levels may be induced by electromagnetic radiation of frequency ν where $\Delta E = h\nu$ and thus the absorption frequency is given by

$$\nu = (\gamma/2\pi)\, \mathbf{B_0} \qquad (9)$$

Thus there are two possible orientations of the nuclear spin for protons and all of the spin ½ nuclei.

The ratio of the populations between these two energy states is given by the Boltzmann equation.

$$N_ß/N_a = \exp(-\Delta E/_{kT})$$

$$= 1 - \Delta E/_{kT} \text{ (as } \Delta E << kT) \text{ (ca. 1 to } 10^5 \text{ for } ^1H) \qquad (10)$$

where N_a and $N_ß$ are the numbers of nuclei in the ground and excited states respectively. Since these energy differences are small, there is only a small excess of nuclei in the a state compared to the ß state. When $N_a > N_ß$ an NMR signal can be observed due the net absorption of energy.

1.3.1 The NMR experiment.

In an NMR experiment we observe the behaviour of an ensemble of spins in a magnetic field. Consider a sample containing only a single type of spin 1/2 nucleus (e.g. the proton in $CHCl_3$). In pictorial vector diagrams the z-axis points along the static magnetic field **(B_0)** direction. The net magnetisation **M** arises from the net sum of all the individual nuclear magnetic moments precessing about the magnetic field direction (z-axis). This net magnetisation is aligned with the applied magnetic field and remains undisturbed (Figure 1.2), in the absence of electromagnetic radiation.

Once this Boltzmann population distribution has been established **M** reaches a stationary equilibrium value $\mathbf{M_0}$.

The second magnetic field $\mathbf{B_1}$, which oscillates at the appropriate radiofrequency in the plane perpendicular to $\mathbf{B_0}$ displaces the magnetisation $\mathbf{M_0}$ away from the z-direction. After the pulse generates $\mathbf{B_1}$, $\mathbf{B_0}$ generates a force on **M** which is a torque that will cause **M** to precess about $\mathbf{B_0}$ at a frequency (?/2p) $\mathbf{B_0}$ Hz. This motion is known as Larmor precession. The component of the precessing magnetisation in the xy-plane will be detected as an NMR signal. The final position of the magnetisation **M** depends upon the length of time (μs) for which the radiofrequency is applied. The angle (?) by which the magnetisation is tipped from the z-axis is called the flip angle or pulse angle.

The orientation of the magnetisation vector **M** can be specified in the coordinate system by the three axial components $\mathbf{M_x}$, $\mathbf{M_y}$ and $\mathbf{M_z}$. The spin system returns to its equilibrium state by relaxation when the pulse $\mathbf{B_1}$ is switched off. The z component $\mathbf{M_z}$ returns to its original position while the x and y components $\mathbf{M_x}$ and $\mathbf{M_y}$ approach zero (Figure 1.3). The motion of the magnetisation vector is described by two different relaxation times $\mathbf{T_1}$ (spin-lattice or longitudinal relaxation time) and $\mathbf{T_2}$ (spin-spin or transverse relaxation time).

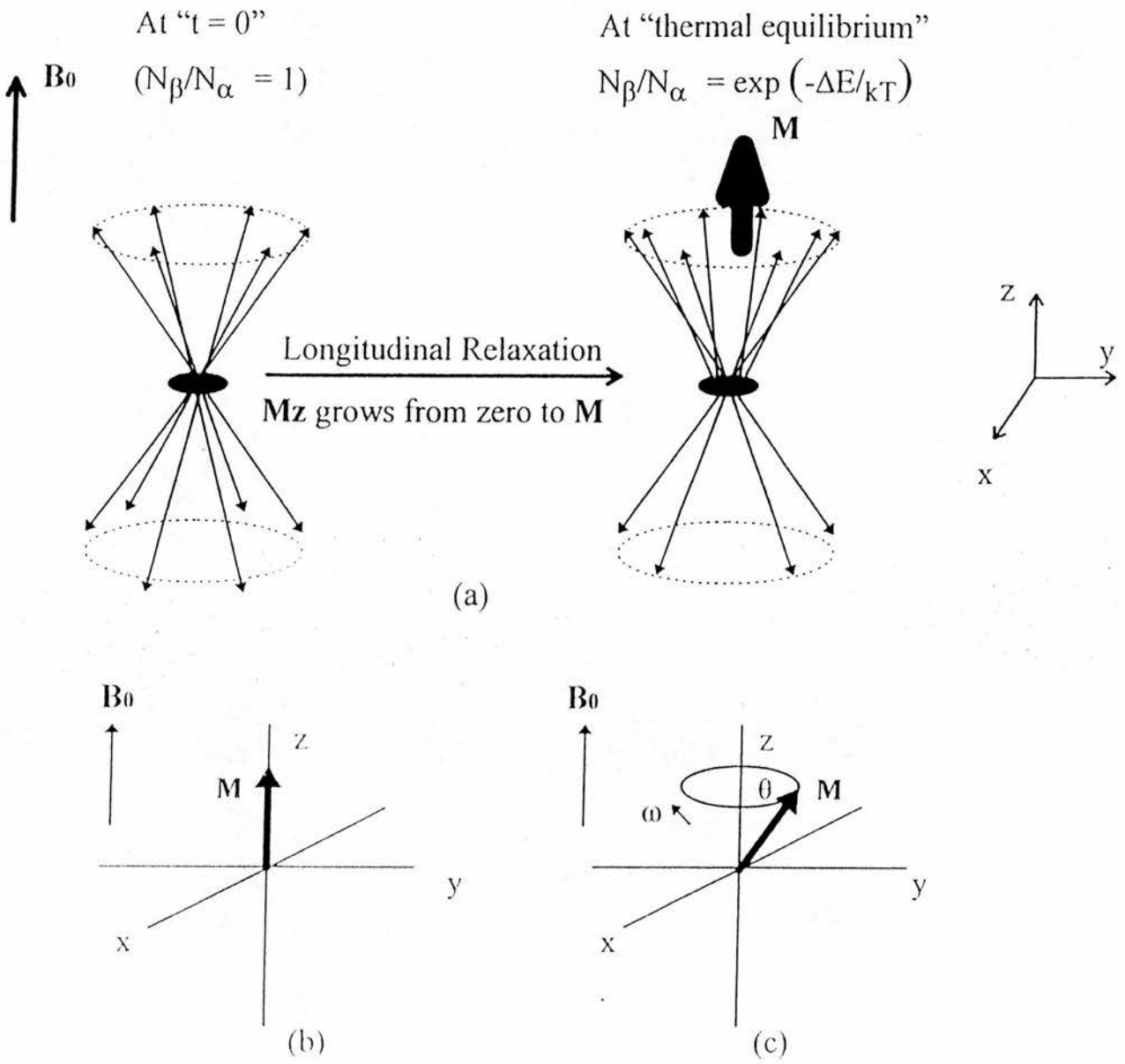


Figure 1.2 Growing of the net magnetisation **M** (a), the **M** at equilibrium (b) and at precession (c).

T_1 is a measure of the rate at which the Boltzmann population distribution is established from any non-Boltzmann situation.

$$\frac{dMz}{dt} = -\frac{(Mz - M)}{T_1} \qquad (11)$$

T_2 is a measure of the rate of decay of the generated NMR signal.

$$\frac{dMx}{dt} = -\frac{Mx}{dt} \qquad (12)$$

$$\frac{dMy}{dt} = -\frac{My}{dt} \qquad (13)$$

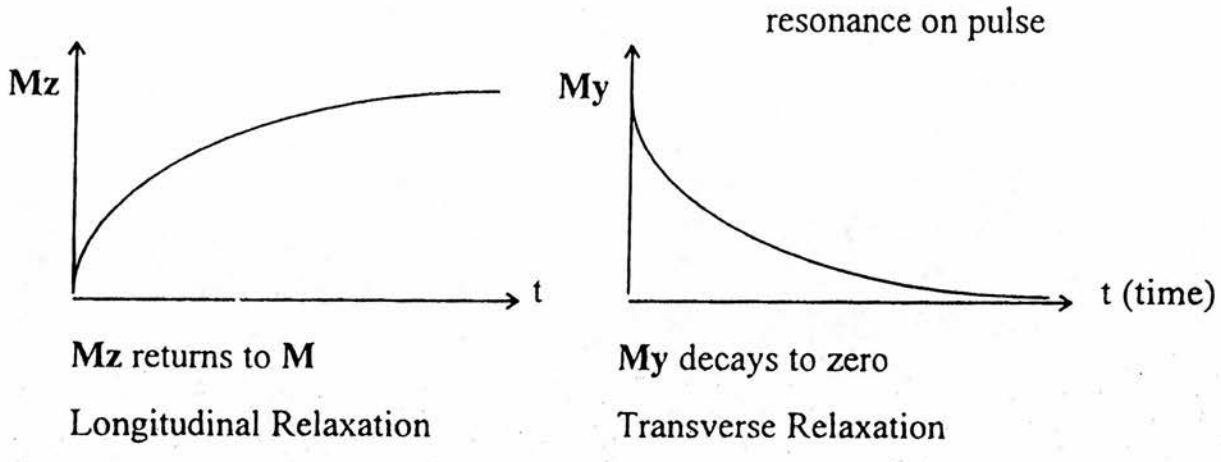


Figure 1.3 Longitudinal and Transverse relaxation of the magnetisation M

1.3.2 Chemical shift

The chemical shift is an inherent property of the molecule. It is caused by the distribution of the electrons in the chemical bonds in the molecule. In a fixed eternal magnetic field $\mathbf{B_0}$ the exact resonance frequency of a nucleus depends on its environment in the molecule. When a molecule is placed in the magnetic field $\mathbf{B_0}$, the magnetic field induces motion in the electron cloud surrounding the nuclei in such a way that a magnetic field opposed to the original $\mathbf{B_0}$ is induced. Thus the electrons shield the nuclei from the applied field $\mathbf{B_0}$. This magnetic shielding is equal to $\sigma\mathbf{B_0}$ where σ is known as shielding constant which is dependent on the electron density surrounding the nucleus. Although the shielding is molecule orientation dependent, molecule reorientation in solution is sufficiently fast for the orientation effect to be averaged.

The chemical shift (δ) as defined below is quoted in units of parts per million (ppm) and will be the same whatever the strength of the field used. The resonance frequency of the signal is measured relatively to a reference or standard compound.

$$\text{Chemical shift } \delta = \frac{\nu_x - \nu_{std}}{\nu_{std}} \times 10^6$$

where $\nu_\chi = ({}^{\gamma}/_{2\pi})\ \mathbf{B_x}$ where the local field experienced by nucleus x is given by $\mathbf{B_x} = \mathbf{B_0}\,(1-\sigma_x)$

1.3.3 Spin-spin coupling.

Often, the individual resonance signals from different atomic nuclei are split into a number of lines. This arises from interactions between magnetic nuclei transmitted through bonding electrons. The splitting of signals can normally be seen in nuclei separated by no more than three bonds but in some cases depending on the geometry of the molecule it can be seen through four or five bonds. The fact that spin- spin coupling is transmitted through chemical bonds makes the coupling constant J, a sensitive indicator for the types of bonds involved and for their spatial orientation in the molecule. The coupling constant is therefore geometry dependent. In complex molecules it can be seen that each nucleus can interact with many others and this can lead to very complicated spin patterns. The spin-spin coupling is field independent and is measured in Hertz (Hz).

When the frequency separation between the signals of the interacting nuclei is large compared to the value of J the appearance of the signal can be predicted by simple rules. A nucleus of spin I splits the resonance of another nucleus (any nucleus) into 2I+1 lines. The number of lines in multiplet, which is called multiplicity M, can be obtained from the following equation:

$$M = 2nI+1 \qquad (14)$$

Here n is the number of equivalent neighbouring nuclei. For nuclei with a magnetic quantum number I=1/2, which include ^{1}H and ^{13}C we can deduce from (14)

$$M=n+1 \qquad (15)$$

For couplings to nuclei with I=1/2 the signal intensities within each multiplet correspond to the coefficients of the binomial series, predicted and obtained from Pascal's triangle:

n=0				1					
n=1			1		1				
n=2		1		2		1			
n=3	1		3		3		1		
n=4	1		4		6		4		1

1.3.4 Solvent suppression

All biofluids have very low concentrations of components. This makes obtaining the Fourier transform ^{1}H NMR spectra of biofluids difficult because the weak solute signals (0.001 - 0.1 M) have to be detected in the presence of a huge signal from the water protons (~ 110 M). A technique widely used to overcome this hurdle is water suppression. The simplest suppression method is continuous weak radiofrequency prior to excitation and acquisition to eliminate the water magnetisation (Hoult, 1976). Irradiation should be gated off during acquisition of data avoid Bloch - Siegert shifts despite the reduction in the level of suppression this entails. The decoupler power should be carefully selected as high decoupler power will give efficient elimination of the solvent resonance but will also saturate protons with nearby chemical shifts. The same applies to irradiation time as long irradiation times will give good saturations but will reduce the efficiency of signal averaging.

Saturation of the solvent peak although highly successful does not achieve total suppression of the peak. Inevitably some protons lie directly under the water signal or are sufficiently close to it in frequency that they do not escape saturation. Any protons bleached in this way are completely invisible in both one and two

dimensional experiments but can sometimes be revealed by applying specific techniques to the spectrum acquisition and editing (Hewage , 1995).

1.4 One dimensional (1D) and two dimensional (2D) NMR experiments

Simple 1D NMR experiments are usually adequate for the study of similar samples from biofluids. The study of biofluids has mostly concentrated in 1D NMR experiments which are simple and have the advantage of acquiring significant amounts of data in high speed. One can resort to 2D experiments in some biofluids like plasma and urine where there is a need for assignment of peaks overlapping in 1D experiments.

The simple presaturation technique is mostly used if all substances have similar molecular weights and similar relaxation times. Single pulse proton NMR spectra are acquired using the pulse sequence D1 - 90^0 - AQ with D1 being a relaxation delay during which the saturation frequency is applied and AQ the acquisition time. In all 1D NMR experiments a preparation time is immediately followed by the data acquisition period.

All 1D NMR spectra have two dimensions: the abscissa which correspond to the frequency axis from which one reads off the chemical shifts and the ordinate which gives the intensities of the signals. In 1D NMR a short intense pulse at a single radio frequency serves to excite all spins from a particular radiofrequency. The response of the sample following the pulse as manifested by the current generated in the receiver coil, is amplified, detected against the frequency of the transmitter, converted from analogue to digital form and then stored in computer memory as amplitude vs time (t_2). The sequence can be repeated after a suitable delay which allows the spins to

return towards equilibrium. FIDs digitised during the acquisition period are combined to improve the signal to noise ratio. Weighting of the averaged time - domain signal with appropriate mathematical functions can be used to increase resolution or signal to noise ratio. The frequency components of the FID are separated by Fourier transformation to give spectral peaks at their characteristic frequencies.

In cell culture media where all molecules are of similar molecular weight and signals are not overlapping the simple presaturation technique is widely used. In the case of more complex biological fluids such as plasma where there are substantial amounts of albumin and lipoproteins, the spectral profile is more complex with broad signals from macromolecules overlapping with sharp signals from small molecules. The use of specific pulse sequences can simplify the spectrum by attenuation of the broad proton signals (Nicholson et al, 1995).

One such sequence which is very useful is the Carr- Purcell- Meiboom-Gill (CPMG) spin echo pulse sequence. This is based on a method for determining T_2 whereby the inhomogeneity contribution is eliminated. In this pulse sequence the 90^0 excitation pulse is not followed by another 90^0 pulse but by a 180^0 pulse. The complete pulse sequence is

$90^0 - \tau - 180^0 - \tau$ (1^{st} echo) $- \tau - 180^0 - \tau$ (2^{nd} echo) .....

The CPMG spin-echo pulse sequence does not J-modulate the pulse sequence. Any loss of signal intensity, during the T_2 relaxation delays by diffusion through field gradients, is minimised by virtue of the short delays employed. In a spin-echo experiment, total spin-spin relaxation delays of <120ms are usually sufficient to attenuate protein resonances by factors of 1000 and hence allow low molecular weight substances to be measured without interference (Lindon et al, 1999).

The use of 2D NMR methods is important as mentioned earlier for spectral assignment purposes. A 2D NMR experiment is recorded in a 2D time space. It differs from 1D by the addition to the pulse sequence of one or more transmitter pulses and one delay (t_1) that is incremented from one acquisition or set of acquisitions to the next. The time span is divided into four periods. (a) a preparation period in which the desired order of coherence is generated. (b) an evolution period during which the spin system evolves under the influence of the chemical shift and scalar couplings. (c) a mixing period during which there is some transfer of coherence and (d) a detection period when the NMR signal is recorded.

As in 1D NMR spectra, the data are digitised as a function of time t_2. The raw data of a 2D NMR experiment consists initially of a series of these FIDs stored in different files each having a different evolution time t_1. FTs are carried out with respect to both time axes t_1 and t_2. The result is spectral intensity as a function of two frequencies m (F_1, F_2). Any signal in the 2D spectrum is defined by two frequencies; $m_{ij}(F_{1i}, F_{2j})$, m being the magnitude of signal at any data point and F_x being the chemical shift dimension. The additional frequency axis allows the correlation of magnetic properties of one nucleus with those of one or more other nuclei that interact with it during the mixing time. Resolution in the F_2 dimension is determined by the digitisation during the t_2 period: resolution in the F1 dimension is limited by the number of incremented t_1 values. Quadrature detection is normally used in acquiring in the t_2 dimension to enhance sensitivity. By obtaining pure-phase spectra, one can avoid the degraded resolution characteristic of peaks in absolute value experiments (Hewage, 1995).

J-resolved (JRES) spectra have been used to simplify the spectra of complex biofluids like plasma and urine (Foxall et al, 1993a). Homonuclear 2D J-resolved spectroscopy is frequently the first 2D experiment to be used in biofluids analysis. The spin-echo pulse used is as follows:

$90^0 - t_1/2 - 180^0 - t_1/2 - AQ(t_2)$.

In practice one records FIDs for a range of different t_1- values at constant increments of a few ms. FT of an individual FID gives a frequency spectrum F2 which contains information on chemical shifts and coupling constants. A second FT with respect to t_1 yields another frequency spectrum F1. This only contains multiplets produced by the couplings and thus gives the coupling constants. All multiplets are tilted relative to the F2-axis by 45^0. All signals of a multiplet belonging to one proton with a chemical shift δ can be made to appear on a line perpendicular to the F2-axis. If this 2D spectrum is then projected onto the F2-axis the resulting spectrum consists only of singlets. The overlapped regions of 1D plasma spectra can be thus more clearly defined (Lindon et al, 1999).

Another helpful 2D experiment used to further define the presence of a number of substances is 2D Homonuclear Correlated Spectroscopy (COSY). COSY provides similar information to the single frequency decoupling experiment which identifies spins that are scalar coupled to one another. In a COSY spectrum, the 1D spectrum lies along the diagonal which runs from bottom left to top right and the off-diagonal elements are present at the intersection of chemical shifts of groups that are J coupled. The pulse sequence used is as follows:

$90^0 - t_1 - 90^0 - AQ(t_2)$

In practice a single COSY spectrum presents a map of the complete spin-spin coupling network in a substance (Nagayama et al, 1980).

1.5 Resonance assignment and quantitation in NMR spectra of biofluids

Usually in order to make assignments in ^1H NMR spectra of biofluids comparison is made with authentic materials or by adding pure substances to the biological fluid. This has served to assign the major peaks in biofluids. Additional confirmation of assignments is sought from the application of 2D NMR methods such as COSY and HMQC (Lindon et al, 1999). The application of 2D J-resolved spectra pulse sequence is important to spread out the coupling patterns of the high number of substances of small molecular weight in a biofluid. But even this often results in a lot of overlapping and further editing is often desirable. This can be achieved by relaxation editing, diffusion editing and multiple quantum filtering (Liu et al, 1998).

One major advantage of NMR spectroscopy as a tool to study biofluids is that measurements can often be made with minimal sample preparation (usually only with the addition of 5- 20% D_2O) and a detailed analytical profile can be obtained on the whole of the biological sample. Minimal preparation also ensures minimal perturbation to the sensitive compounds in the biofluid (Nicholson et al, 1989). Other advantages are that it can be fast with spectra being obtained in 5 to 15 minutes and also that it can provide detection of all the substances in the biofluid providing that they are present above the detection limit.

Detailed ^1H NMR spectroscopic data exist for most biological fluids and have been obtained using the above mentioned methods (Lindon et al, 1999, Fan et al, 1986, Sze et al, 1994). Table 1.1 gives data for the substances detected in plasma.

Most work done in this thesis was based on reconfirming data from that Table and then proceeding with the quantitation of the concentrations in the biofluids.

Table 1.1

Assignment of peaks to substances from spectra of human plasma. The peaks marked with an asterisk (*) were used for quantitation of the corrresponding analyte.

	Assignment	$\delta(^1H)$	Multiplicity
Isoleucine	δ-CH_3	0.94	t
Leucine *	δ-CH_3	0.96	t
Leucine	δ-CH_3	0.97	t
Valine	CH_3	0.99	d
Isoleucine*	β-CH_3	1.01	d
Valine *	CH_3	1.04	d
Ethanol *	CH_3	1.11	t
Isobutyrate	CH_3	1.13	d
β-Hudroxybutyrate *	CH_3	1.20	d
Isoleucine	γ-CH_2	1.26	m
Fucose	CH_3	1.31	d
Lactate *	CH_3	1.33	d
Threonine *	CH_3	1.34	d
Alanine *	CH_3	1.48	d
Lysine	γ-CH_2	1.48	m
Isoleucine	γ-CH_2	1.48	m
n-Butyrate	β-CH_2	1.56	d
Adipate	CH_2	1.56	m
Leucine	CH_2	1.71	m
Lysine	δ-CH_2	1.73	m
Ornithine	γ-CH_2	1.81	m
Citrulline *	β-CH2	1.88	m
N-Acetylglutamate	β-CH_2	1.89	m
γ-Amino-n-butyrate	β-CH_2	1.91	m
Lysine	β-CH_2	1.91	m
Arginine *	β-CH_2	1.93	m
Acetate*	CH_3	1.95	s
Isoleucine	β-CH	1.98	m
N-Acetyl groups	CH_3	2.02	s
N-Acetyl groups	CH_3	2.05	s
Glutamate	β-CH_2	2.10	m
Glutamine *	β-CH_2	2.14	m
Methionine *	S-CH_3	2.14	s
n-Butyrate	α-CH_2	2.16	t
Methionine	β-CH_2	2.16	m

Acetone *	CH_3	2.23	s
Valine	β-CH	2.28	m
Acetoacetate *	CH_3	2.29	s
β-Hydroxybutyrate	CH_2	2.31	ABX
Glutamate *	γ-CH_2	2.36	m
Oxalacetate	CH_2	2.38	s
Pyruvate *	CH_3	2.38	s
β-Hydroxybutyrate	CH_2	2.41	ABX
Succinate	CH	2.43	s
Carnitine	CH_2(COOH)	2.44	dd
α-Ketoglutarate	γ-CH_2	2.45	t
Glutamine	γ-CH_2	2.46	m
Glutamate	γCH_2	2.50	m
Methylamine *	CH_3	2.54	s
Citrate *	1/2CH_2	2.67	AB
Methionine	S-CH_2	2.56	t
Aspartate *	β-CH_2	2.68	ABX
Dimethylamine *	CH_3	2.72	s
Dimethyglytcine	CH_3	2.78	s
Citrate	1/2CH_2	2.80	AB
Aspartate	β-CH_2	2.82	ABX
Asparagine	β-CH_2	2.86	m
Asparagine	β-CH_2	2.96	m
α-Ketoglutarate	β-CH_2	3.01	t
Lysine	ε-CH_2	3.03	t
Creatine *	CH_3	3.04	s
Creatinine *	CH_3	3.05	s
Tyrosine	CH_2	3.06	ABX
Phenylalanine	β-CH_2	3.13	m
Histidine	β-CH_2	3.14	ABX
Tyrosine	CH_2	3.20	ABX
Choline	$N(CH_3)_3$	3.21	s
β-Glucose	C-H2	3.24	dd
Histidine	β-CH_2	3.25	ABX
Trimethylamine-N-oxide *	$N(CH_3)_3$	3.27	s
Taurine	CH_2SO_3	3.25	t
Betaine	$N(CH_3)_3$	3.27	s
Phenylalanine	β-CH_2	3.28	m
Myo-Inositol	H5	3.28	t
Tryptophan	CH_2	3.31	ABX
Proline	δ-CH_2	3.33	m
β-Glucose	C-H4	3.40	t
α-Glucose	C-H4	3.41	t
Proline	δ-CH_2	3.42	m
Carnitine	NCH_2	3.43	m
Taurine *	NCH_2	3.43	t
Acetoacetate	CH_2	3.45	s

β-Glucose	C-H5	3.47	ddd
β-Glucose	C-H3	3.49	t
Tryptophan	CH_2	3.49	ABX
Choline	NCH_2	3.52	m
α-Glucose	C-H2	3.53	dd
Glycerol	CH_2	3.56	ABX
Myo-Inositol	H1/H3	3.56	dd
Glycine *	CH_2	3.57	s
Sarcosine	CH_2	3.61	s
Ethanol	CH_2	3.61	q
Valine	α-CH	3.62	d
Myo-Inositol *	H4/H6	3.63	dd
Glycerol	CH_2	3.65	ABX
Isoleucine	α-CH	3.68	d
α-Glucose	C-H3	3.71	t
β-Glucose	C-H6	3.72	dd
Leucine	α-CH	3.73	t
α-Glucose	C-H6	3.74	m
Lysine	α-CH	3.76	t
Glutamine	α-CH	3.77	t
Glutamate	α-CH	3.77	t
Glycerol *	CH	3.79	ABX
Alanine	CH	3.79	q
Ornithine	α-CH	3.79	t
α-Glucose	C-H6	3.84	m
α-Glucose	C-H5	3.84	ddd
Methionine	α-CH	3.86	t
β-Glucose	C-H6	3.90	dd
Betaine	CH_2	3.90	s
Creatine	CH_2	3.93	s
Tyrosine	CH	3.94	ABX
Hippurate *	CH_2	3.97	d
Histidine	α-CH	3.99	ABX
Phenylalanine	α-CH	4.00	m
Creatinine	CH_2	4.06	s
Choline	OCH_2	4.07	m
Lactate	CH	4.12	q
Proline	α-CH	4.14	m
β-Hydroxybutyrate	CH	4.16	ABX
Theonine	β-CH	4.26	ABX
β-Galactose	CH1	4.53	d
β-Glucose	CH1	4.64	d
Water	H_2O	4.79	s
α-Glucose *	CH1	5.23	d
Urea *	NH_2	5.78	s
Tyrosine *	H3/H5	6.91	d
3-Methylhistidine	H4	7.01	s

1-Methylhistidine	H4	7.05	s
Tyrosine	H2/H6	7.20	d
Phenylalanine *	H2/H6	7.33	m
Phenylalanine *	H4	7.38	m
Phenylalanine *	H/H5	7.43	m
3-Methylhistidine	H2	7.61	s
1-Methylhistidine	H2	7.77	s
Histidine *	C2H	7.83	s
Formate	CH	8.46	s

We have used ^{1}H NMR spectroscopy to quantitate the amounts of different substances present in the biological fluids studied. To ensure that this analysis is quantitative some assumptions were made. All spectral lines of the compounds studied were narrow compared to chemical shift differences. To obtain quantitative results we integrated the areas under the lines bearing in mind that electronic integrators under the best conditions are accurate to 2% of full scale measurement. The lines studied were clearly defined and the signal to noise ration was high. There were no major satellite peaks present and those seen did not overlap with peaks that were studied. Spinning side bands were added to the main line band for the integration. By gating the decoupler off during the delay period the Nuclear Overhauser Effect (NOE) was eliminated. Finally the lines studied for the quantitative measurements were not produced by quickly exchangeable protons.

Chemical shifts were referenced internally to the singlet methyl resonance of Sodium 3-(trimethylsilyl 2,2,3,3-$^{2}H_4$) -1 propionate (TSP) at zero ppm. Quantitation of compounds present in the samples was achieved by two alternative means. (a) Integrals were measured relative to that of a known quantity of TSP (10 mg/ml) present as an internal standard to the solution (Foxall et al, 1993b). (b) When signals were partially overlapped, peak height measurements were used, taking into account the appropriate coupling pattern of intensities of the non-overlapped lines.

1.6 NMR Spectroscopy of blood plasma

Blood plasma is a constituent of normal blood which is obtained from untreated whole blood after the addition of an anticoagulant (trisodium EDTA or lithium heparin) and after centrifugation in order to discard all the cellular constituents of blood. Plasma consists mainly of water in which there are several distinct domains. These include an essentially isotropic, free-solution environment, which contains colloidal structures and aggregates of macromolecules such as lipids and proteins. It also contains a large number of inorganic and low molecular weight solutes.

The physicochemical complexity of plasma is expressed in its ^{1}H NMR spectra by the range of linewidths of the signals. This means that biochemical information is difficult to extract and most of the previously mentioned techniques are routinely used to extract maximum information from the spectra. Eventually, ^{1}H NMR spectra of plasma can provide a plethora of biochemical information on both low molecular weight metabolites and macromolecular structure and organisation. The usefulness of ^{1}H NMR spectra of plasma has been the central theme of recent reviews (Nicholson et al 1995; Lindon et al, 1999). It has been noted that there are difficulties in obtaining quantitative determinations in blood plasma but new techniques have shown that there is potential to overcome that, if every substance is considered separately. For example protein binding of lactate makes about 30% of its concentration NMR invisible initially but after addition of ammonium chloride this can be overcome. A similar effect is reported for acetoacetate. (Bell et al, 1988a).

Although ^{1}H NMR spectra of plasma have been used to study metal complexes (Nicholson et al, 1983), protein - ligand binding (Bell et al, 1988a),

lipoproteins (Ala-Korpela 1995) and to evaluate the molecular diffusion coefficients of plasma substances (Liu et al, 1995), their main role is to characterise different pathological states.

1.7 NMR spectroscpy of plasma in pathological states

1.7.1 Malignant diseases

Excitement was generated when a paper reported that by using ^{1}H NMR spectra one could differentiate between patients with malignant tumours and normal subjects. (Fossel et al, 1986). This was based on the study of specific signals arising from lipoproteins. Although numerous researchers have attempted to reproduce the original findings, they have been unable to do so. Other factors, including diet (Bell et al, 1988b), age and gender (Engen et al, 1991) and hyperlipidaemia (Mims et al, 1989) have been found to cause similar changes in this signal pattern. Other lipoprotein bands especially from fucosilated moieties have also been studied by ^{1}H NMR spectroscopy (Mountford et al, 1987). Other studies of ^{1}H NMR spectra of blood plasma in malignant disease have included general studies (Ötvos et al, 1987), testicular cancer (Höfeler et al, 1989) and lung cancer (Schumacher et al, 1990), comparison of NMR and chromatographic methods (Chmurne et al, 1988), the effects of plasma sialic acids (Kriat et al, 1992) and the effect of dyslipoproteinaemias (Herring et al, 1989).

1.7.2 Transplantation

Heart transplantation has been studied most. Most studies concentrated on providing evidence of rejection after transplantation. Studies on the same signals of

lipoproteins reported accurate identification of rejection (Eugene et al, 1988, 1991). Another study found a correlation between the areas of two glycoprotein signals and the isovolumetric left ventricular relaxation time post transplantation (Pont et al, 1991). In kidney transplantation a study was performed to look at these same lipoprotein signals but the results were negative (Eugene et al, 1989) .

1.7.3 Malaria

One study exists looking at inpatients seropositive to Plasmodium which showed higher lactate concentrations than controls. It also showed that there were significant differences in the same signals of lipoproteins as previously (Nishina et al, 1988).

1.7.4 Diabetes mellitus

In ^{1}H NMR spectra there are marked elevations in the concentrations of all three ketone bodies i.e. acetoacetate, acetone and ß-hydroxybutyrate and glucose in diabetic patients. The levels of lactate, valine and alanine were also measured and there was a good correlation in concentrations measured by NMR and standard biochemical methods. Concentrations of VLDL lipoproteins and chylomicrons were found to be decreased. In non insulin dependent diabetics the level of triglycerides observed was high, decreasing after better diabetic control (Bell et al, 1989).

1.7.5 Renal diseases

There has been a study comparing plasma from patients on dialysis, patients with early renal failure and controls (Grasdalen et al, 1987). Acetate accumulation

was seen in patients in dialysis when plasma components were monitored. TMAO was high in patients with renal failure. In another study TMAO concentrations correlated with the degree of renal failure (Foxall et al, 1993c). Dimethylamine and trimethylamine were shown to be elevated along with lactate and creatinine. In the same study, plasma from patients with chronic renal failure and controls were compared and metabolic abnormalities were seen (Foxall et al, 1993c). Metabolic abnormalities were also seen in the plasma of patients with uraemic syndrome in another study (Foxall ,1993d).

1.7.6 Liver diseases

Liver diseases have been the centre of many NMR studies. Most of them were performed in what is known as the *in vivo* setting. This means that instead of using body fluids as samples to look at metabolites, whole organisms are placed in an NMR scanner. The resulting spectra have the advantage of looking at the structure of a whole organ and identify differences in metabolic activity between parts of the organ. The biggest disadvantage of this technique has been its limitation by the study of a living organism and the subsequent use of low power magnets normally less than 3.0 T. The spectra acquired have a very low resolution and a lot of overlapping and it is almost impossible to quantitate concentrations directly from the spectrum. Another setback of the method is that, as it uses small voxels to acquire spectra, a considerable amount of time is spent for one experiment and this can be up to 2 hours.

A number of *in vitro* studies have been conducted looking at other nuclei than hydrogen in particular carbon and phosphorus. Phosphorus NMR spectroscopy has an advantage in producing data reflecting the bioenergetics of the cell studied but

apart from that it is difficult to obtain information about other key metabolic pathways which do not contain high energy phosphorus bonds. Carbon NMR spectroscopy can be effective but the mean time for the acquisition of one set of spectra is a lot higher than with hydrogen and the spectra are a lot more complex to analyse.

There are few studies in liver disease that have used ^{1}H NMR Spectroscopy. Although most of them were set out to answer specific questions in a general context of research they definitely show the potential of the technique. Two studies have used hydrogen and carbon NMR to study the performance of specific reactions in the Kreb's cycle in rat livers (Petersen et al, 1994, 1995). Other studies have looked at liver metabolism in the context of cold hypoxia (Lockett et al, 1996; Churchill et al, 1997; So et al, 1998). One study has looked at the intracellular metabolites of cultured liver cells (De Loecker et al, 1993) and another study has looked at liver slices from animals of different species (Fuller et al, 1994). One study still in abstract form has looked at some metabolites in the plasma of patients suffering from cirrhosis caused by primary biliary cirrhosis before and after Transjulular Intrahepatic Portosystemic Stent Shunt (TIPSS) and concluded that there were differences between controls and patients before and after TIPSS (Jalan et al, 1995a). No systematic study was performed before in either the area of cultured primary hepatocyte by NMR and how culture conditions affect their performance or in patients suffering from acute of chronic liver failure.

1.8 Liver anatomy

1.8.1 Gross clinical anatomy.

The liver is the largest internal organ in the body. It weighs about 1.5 (1.2-1.8) kg and is roughly wedge-shaped. It is situated in the right hypochondrium and occupies most of the right upper quadrant of the abdomen. Its upper border lies between the fifth and sixth ribs and its inferior border extends from the left extremity of the upper border to the tip of the tenth rib.

The liver has a diaphragmatic surface to which the falciform ligament is attached, and a visceral surface which contains the porta hepatis where the hepatic artery and portal vein are entering the liver and the common hepatic duct leaves the liver, the gallbladder fossa and the inferior vena cava (Figure 1.5).

The parenchymal mass of the liver is divided into two main lobes, the right which is larger and the left. The right lobe also contains the quadrate, the caudate and Riedel's lobes (Figure 1.5).

The blood supply to the liver is via the hepatic artery which supplies 25% of the total blood flow and 50% of the oxygen and the portal vein which supplies 75% of the blood flow and the other 50% of the oxygen supply (Figure 1.5).

1.8.2 Microscopic anatomy and function

The functional unit of the liver is the hepatic acinus (Rappaport, 1958). This is defined as a mass of parenchyma that has a single blood supply by hepatic arterial and portal venous branches in the smallest portal tract. The portal tracts are well defined and contain a portal vein radicle, an hepatic arteriole and a bile ductule. A

simple acinus contains a portal triad, hepatocytes that are the predominant liver cells, sinusoids which are the capillary vessels of the liver and two hepatic veins situated at the edges of the acinus. The acinus looks superficially homogeneous but in fact it is heterogeneous in respect of the hepatocytes, the sinusoids, the perisinusoidal cells and the extracellular matrix.

The hepatocytes near the portal triad are said to belong to zone 1. They are well supplied in oxygenated blood, and in the normal well fed state their enzymatic armoury is geared towards catabolism of fatty acids and gluconeogenesis. They are thought to be more resistant to injury that the cells in zone 3 which is near the hepatic vein.

Hepatocytes in zone 3 are not well supplied in oxygenated blood and their main source of energy is glycolysis. They are also more active in drug metabolism (Jungermann et al, 1982) (Figure 1.6).

Kupffer cells, the liver macrophages are found in the sinusoids particularly in zone 1. Hepatic stellate cells found in the space of Disse, in the perisinusoidal space are most numerous in zone 3. They are important in the production of connective tissue components in hepatic repair

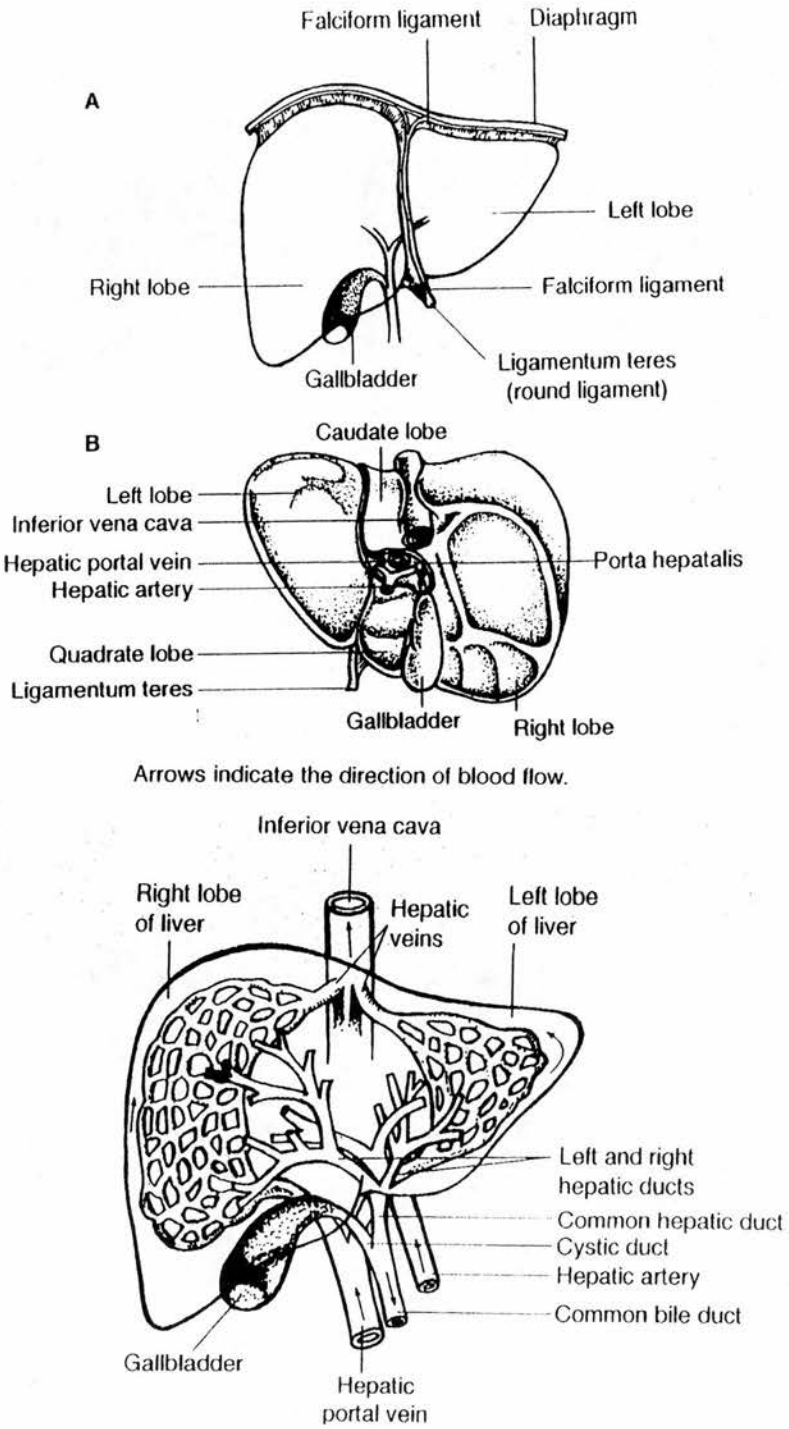


Figure 1.4 Liver lobes shown from the diaphragmatic surface (A) and the peritoneal surface (B), and a schema of the hepatic vascularisation (C)

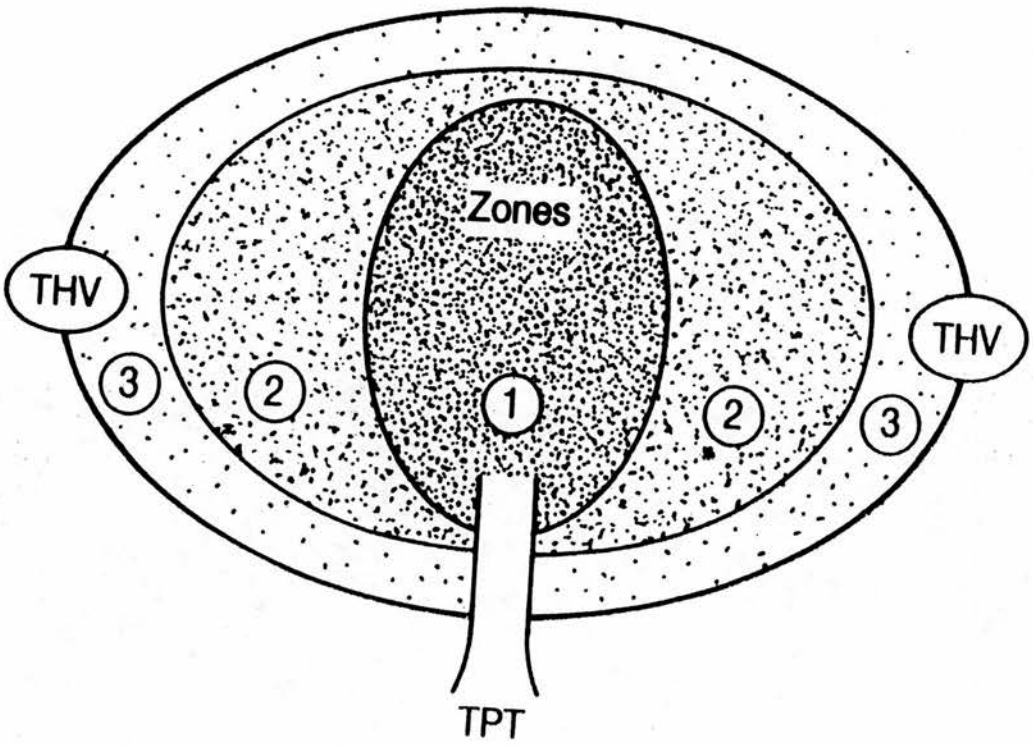


Figure 1.5 The zonal subdivision of the hepatic acinus. TPT= terminal portal tract, THV=terminal hepatic vein

1.9 Liver physiology and biochemistry

1.9.1 Carbohydrate metabolism.

The liver acts as the glucostat of the body. The maintenance of the blood sugar is one of the most important functions of the liver. All hepatocytes are capable of metabolising glucose. In the well fed state glucose is stored in the form of glycogen. If the glycogen stores are full and glucose is still coming into the liver from the portal blood then glucose is diverted into fatty acid synthesis. Fatty acids are then esterified and stored as triacylglycerols in the liver itself or in the adipose tissue.

In starvation or simply in between meals the liver uses either glucose or fatty acids to extract energy. Glucose can be oxidised either aerobically or anaerobically. Both paths have a common metabolic pathway known as glycolysis. One molecule of glucose is transformed to two molecules of pyruvate.

If enough oxygen is present then the aerobic metabolism is active. Pyruvate is fed into the Krebs cycle and it is completely oxidised to form three molecules of carbon dioxide. Energy is conserved in ATP molecules and water is also formed. ATP can then be used to provide energy for biological work. The net yield from one molecule of glucose is 38 molecules of ATP (Figure 1.7).

If the hepatocytes are operating under hypoxic conditions then the anaerobic metabolism becomes active. Pyruvate is transformed to lactate which is the end product of the pathway. Anaerobic oxidation of one molecule of glucose provides only 3 molecules of ATP. Lactate as a by-product of anaerobic metabolism is

excreted from the hepatocytes as it can cause rapid changes in their physiological pH (Figure 1.7).

Hepatocytes are capable of metabolising all the simple sugars. Galactose can be metabolised to glucose with consumption of ATP. Fructose can also be phosphorylated with consumption of ATP and can enter the pathway of glycolysis.

Hepatocytes are not only capable of breaking down glucose, they are also capable of synthesising glucose. Gluconeogenesis is unique to the liver and kidney cortex. The main substrates for gluconeogenesis are lactate and amino acids but glycerol can be used as well. The bulk of lactate would normally be imported from the muscles who tend to use quite extensively glucose anaerobically for their energy requirements (Figure 1.7).

Glycogen is a unique form of glucose storing molecule. Approximately 80 g of glycogen can be stored in the liver. This is stored very temporarily as the glycogen would be the first line of energy storage that would normally be used if need arises. Glucogenolysis is a quick and efficient way to extract energy from stored glucose. Glycogen molecules are cleaved to their individual glucose molecules and they then enter glucolysis (Figure 1.7).

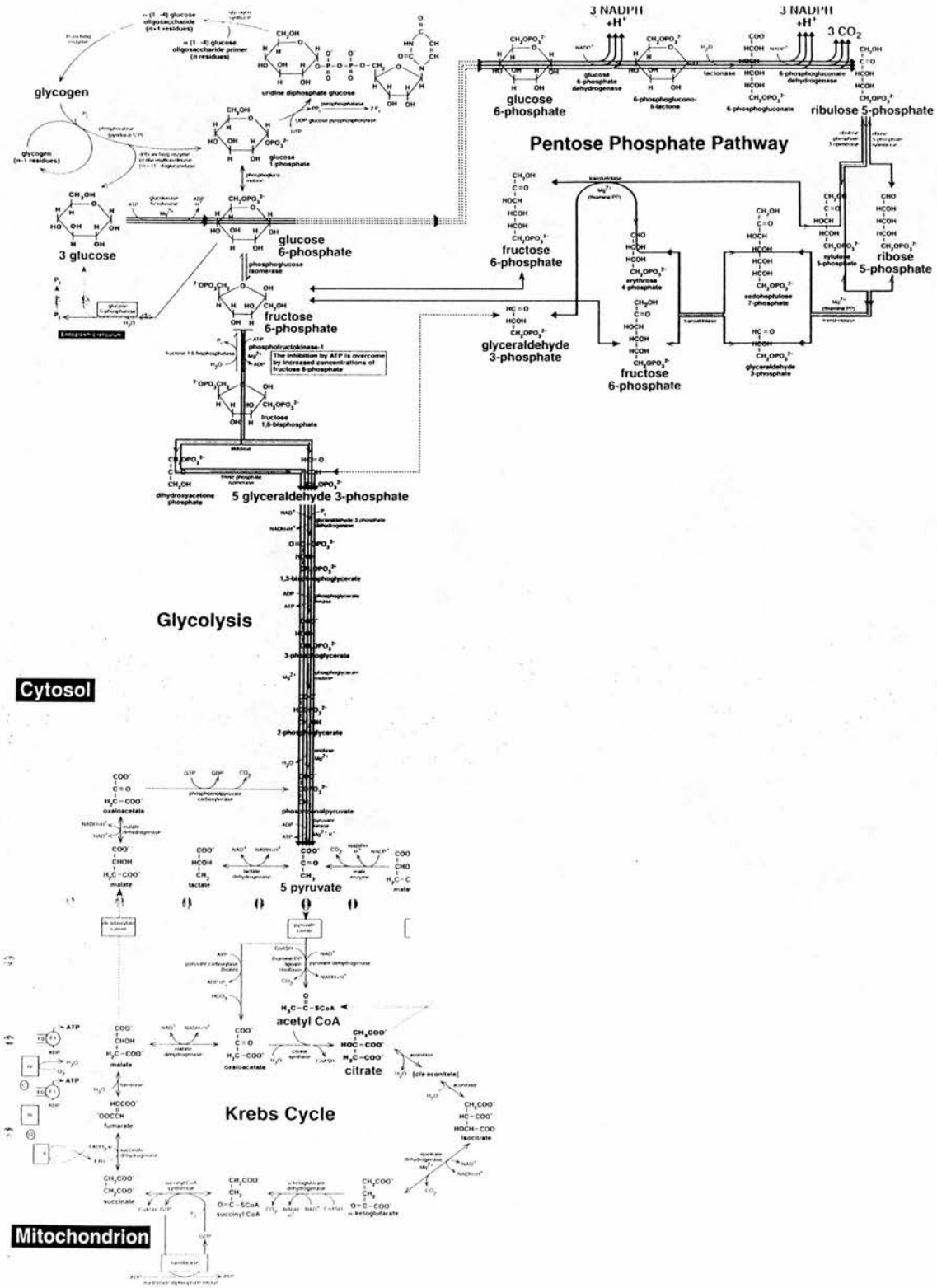


Figure 1.6 Glycolysis and the pentose phosphate pathway

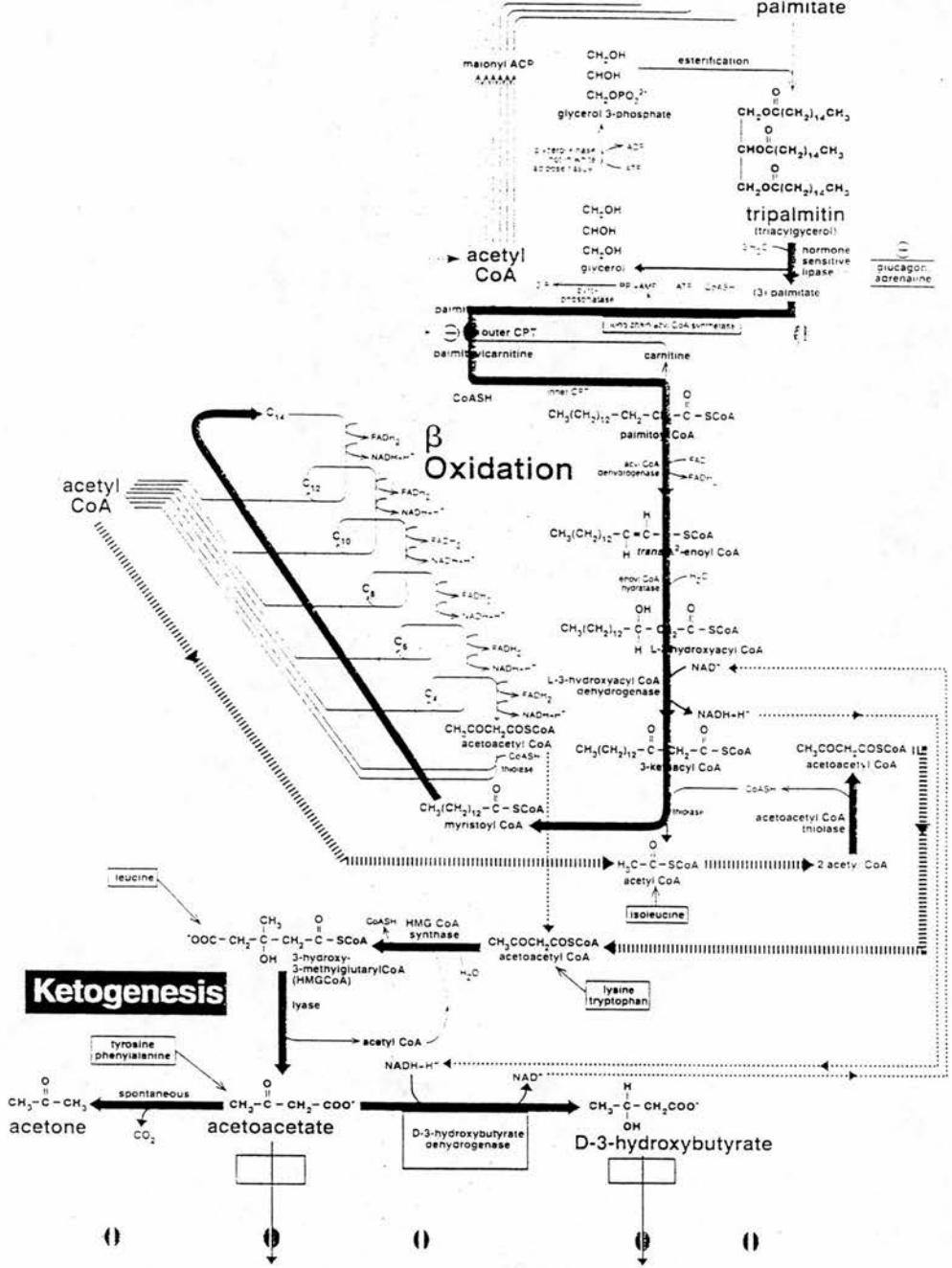


Figure 1.7 Fatty acid oxidation and ketogenesis

1.9.2 Lipid metabolism.

The liver plays a pivotal role in lipid metabolism. It can form *de novo* triglycerides and it can also oxidise fat. It also plays a major role in the metabolism of the lipoproteins which carry the esterified lipids into the blood stream. The liver produces very low density lipoproteins (VLDLs) and high density lipoproteins (HDLs). It also degrades intermediate density lipoproteins (IDLs) and low density lipoproteins (LDLs)

Triglycerides are the final product of fat synthesis. They are composed of three molecules of free fatty acids and a molecule of glycerol. Fatty acids are produced in the liver from the excess of glucose. They are esterified with glycerol in the cytoplasm of the hepatocytes and are then incorporated into the lipoproteins to form very low density lipoproteins (VLDLs). Oxidation of triglycerides is also taking place in the hepatocytes when the cells need energy. Triglycerides are cleaved in the cytoplasm to form glycerol and free fatty acids. The latter move then into the mitochondria of the cells and they undertake there β-oxidation which produces ATP and acetyl-CoA. Acetyl-CoA can then enter the Krebs cycle and produce more ATP (Figure1.8).

During starvation, severe prolonged exercise or uncontrolled diabetes acetyl-coA cannot enter the Krebs cycle. It is then diverted into ketogenesis to form the ketone bodies acetoacetate, β-hydroxybutyrate, and acetone which is spontaneously produced from acetoacetate. All three are collectively known as the ketone bodies. The liver is particular at that point as it normally has an excess of acetyl-CoA which

is coming mainly from the adipose tissue. The liver tissue then forms ketone bodies, mainly acetoacetate which then exports to other tissues (Figure 1.8).

1.9.3 Protein and amino acid metabolism.

The liver is the principal site of synthesis of all the circulating proteins apart from the γ-globulins. It receives amino acids from the intestine and the muscle and uses them to form proteins. By controlling gluconeogenesis and transamination , the other two metabolic pathways that can consume amino acids, it regulates the level of protein in the plasma.

Albumin is the major protein contained in the plasma. It has a half-life of 16-24 days and 10-12 g are synthesised daily. Reduced synthesis of albumin over prolonged periods of time results in hypoalbuminaemia which is a characteristic of chronic liver disease.

Transport or carrier proteins such as transferrin or ceruloplasmin and other proteins like α_1-antitrypsin and α- fetoprotein are also synthesised by the liver. It can also produce all coagulation factors apart from factor VIII and components of the complement system.

The liver also breaks down proteins, into their constituent amino acids that can either be rearranged to form new types of proteins or can be further degraded to produce urea which is then transported to the kidneys for excretion.

Amino acid metabolism is an essential element of hepatocyte physiology. Liver cells can synthesise all the non-essential amino acids. Tyrosine is formed from the essential amino acid phenylalanine. Serine, glycine and cysteine are made from intermediates formed in the glycolytic pathway. Glutamate, glutamine, proline and

arginine are formed from the Krebs cycle intermediate α-ketoglutarate. Aspartate is formed from oxaloacetate, another intermediate of the Krebs cycle and asparagine is synthesised by transamidation from glutamine (Figure 1.9).

Amino acids play a pivotal role in the production of substrates in the hepatocyte. As well as being the building blocks of proteins they can be used in starvation by the hepatocytes to produce glucose. Amino acids can be transformed to pyruvate (i.e. methionine, alanine, tryptophan), α-ketoglutarate (i.e. glutamate, arginine) or 2- phosphoglycerate (i.e. phenylalanine, aspartate, serine) and those substrates can be incorporated into the gluconeogenetic pathway (Figure 1.10).

Oxaloacetate and pyruvate formed from amino acids can be intermediates in the fatty acid synthesis, so amino acids can be used for triglyceride synthesis. Via the 1-carbon pool and folate metabolism amino acids can be used for the synthesis of purines and pyrimidines which are the building blocks for nucleotides.

Overall each amino acid in the hepatocyte can be broken down and the substrates formed in the process can feed almost every metabolic cycle operating in the hepatocytes.

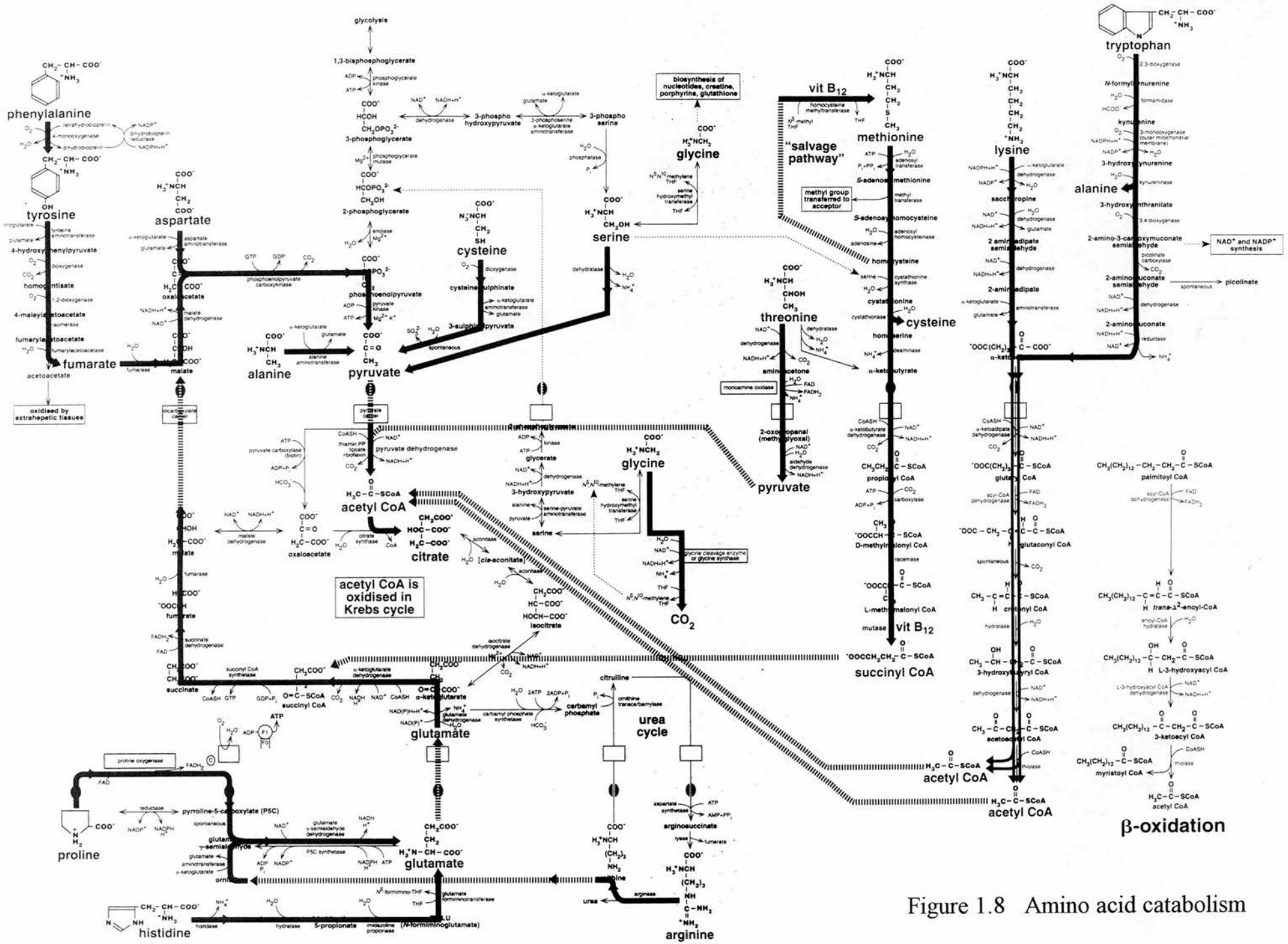


Figure 1.8 Amino acid catabolism

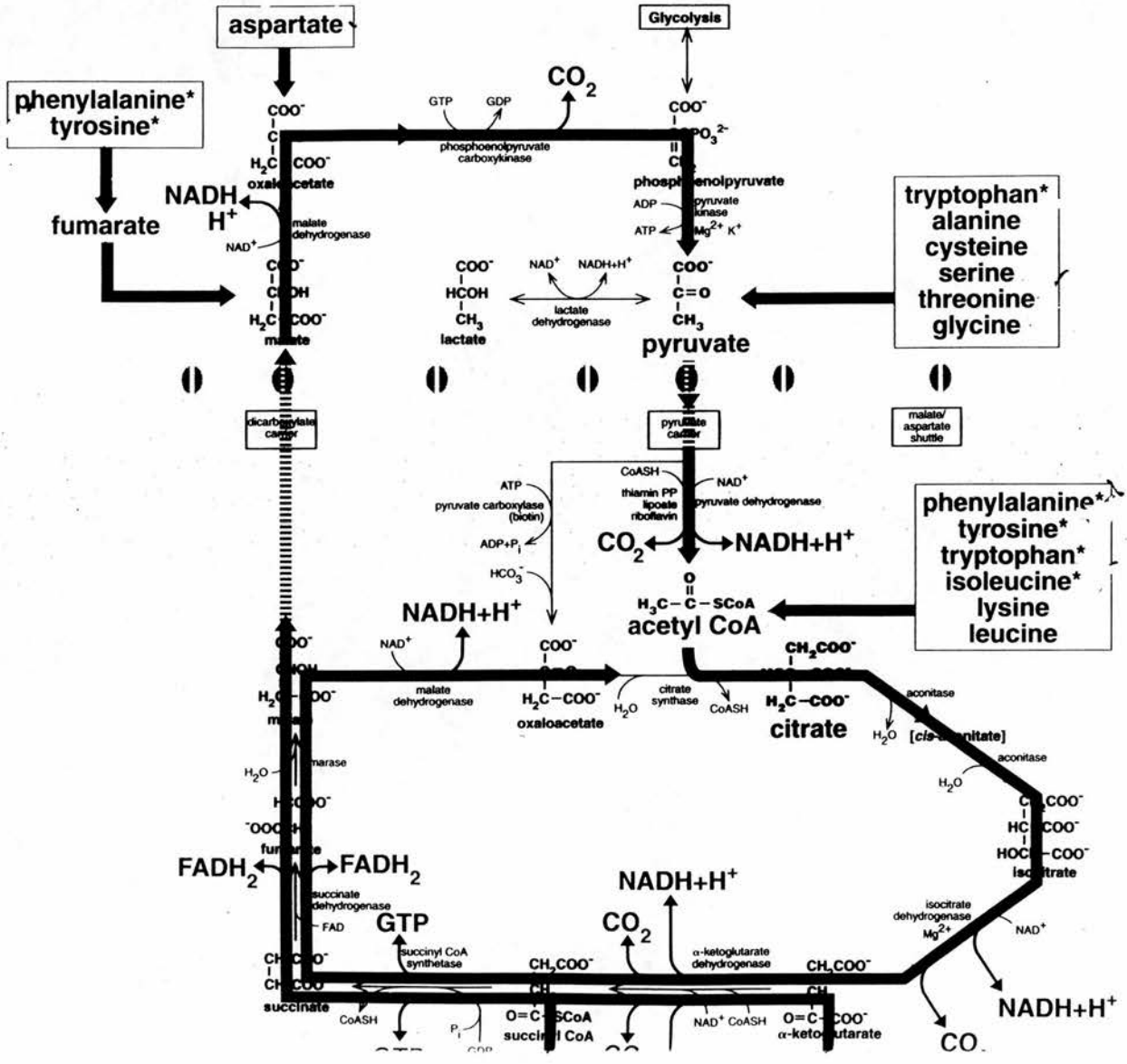


Figure 1.9 Ketogenic and gluconeogenic amino acids and their point of entry into the glycolytic pathway

1.10 Liver cirrhosis

Cirrhosis is the final common pathway of most chronic liver injuries. It is a combination of hepatic fibrosis and nodular regenerative hyperplasia. Although there are many causes of chronic liver injury only a small number of those, account for the majority of cases. In the developed world alcohol accounts for 50 -60% of all cases and is the most important cause. In Africa and Asia the most important cause is hepatitis B infection which accounts for around 10% of cases in the Western world. In recent years hepatitis C is increasingly recognised as an important cause of cirrhosis world-wide and accounts for more than 10% of cases of cirrhosis in the developed world. Primary biliary cirrhosis and autoimmune hepatitis are important although infrequent causes of cirrhosis as the patients suffering from them are very good candidates for liver transplantation. Metabolic causes such as Wilson's disease and haemochromatosis are important causes since early recognition can lead to effective treatment. Although our understanding of the pathogenesis of cirrhosis has improved and new causes are increasingly recognised, a significant minority (5-10%) are still labelled as cryptogenic cirrhosis (Hayes, 1995).

The incidence of cirrhosis in most parts of the world is not known and must vary considerably. In the Western world it is estimated that the incidence of cirrhosis is around 15 -25 in 100000 population (Saunders et al, 1982). During the last thirty years the incidence has tripled reflecting mainly the increasing incidence of alcoholic liver disease.

1.10.1 Pathogenesis

Chronic liver injury resulting in the development of inflammation and subsequent progression to cell death is the common path for the development of fibrosis. The mechanisms that result in death of hepatocytes are complex and differ depending on the etiologic agent. Direct cytotoxic effect of the causative agent, immunological mechanisms with neoantigen formation and free radicals production are some examples.

It has emerged that the role of stellate cells in the process of fibrosis is pivotal. These cells are possibly activated by cytokines, start to proliferate and synthesise collagen and laminin. This results in production of extracellular matrix around the sinusoids and as a result the hepatic sinusoids are capillarised. Increases of all types of collagen occur but it seems that type I and type IV collagen are preferentially produced. (Friedmann et al, 1993).

Simultaneously, hepatocytes lose their microvilli and the sinusoidal fenestrations are occluded. This results in reduced hepatic uptake of water and solute from the circulation aggravating the liver function as cirrhosis progresses (Villeneuve et al, 1996)

The production of collagen leads to production of large bundles of fibrous tissue and fibrosis of the liver. This causes disruption of the liver framework and simultaneous hepatocellular regeneration causes formation of nodules.

1.10.2 Pathology

The whole mass of the liver should be involved in fibrosis and regeneration in order for the liver to qualify as cirrhotic. Regenerated nodules are surrounded by connective tissue. But in the nodule, the normal liver appearance is preserved. Other features that can be encountered in the parenchyma of a cirrhotic liver are, variation in the size of hepatocytes, increased thickness of the liver cell plates and abnormal vasculature.

Two main categories of cirrhosis have been traditionally laid out depending on the nodule size. In micronodular cirrhosis the size of the nodules is less than 3mm in diameter, while in macronodular cirrhosis it is typically more than 3mm in diameter. When both sizes are present cirrhosis is then described as mixed (Anthony et al, 1977).

1.10.3 Clinical Features

These are divided in hepatic and portal venous features and extrahepatic features (Hayes,1995). Abdominal pain, palpable liver and hepatomegaly, palpable spleen and splenomegaly together with dilated superficial collaterals radiating from the ombilicus are the commonest hepatic and portal signs and symptoms.

The commonest extrahepatic features are manifested on the skin and in the circulation. Skin signs include jaundice which is a sign of advanced cirrhosis, pigmentation, finger clubbing, Dupuytren's contractures, bruising and purpura.

Vascular abnormalities include spider telangectasias and epistaxis. A hyperdynamic circulation is characteristic of cirrhosis. Patients have tachycardia, increased cardiac output, low blood pressure and low peripheral vascular resistance. Splanchnic circulation is also affected on top of the abnormalities in the systemic

circulation. There is increased splanchnic blood flow, portal hypertension and increased flow in the portasystemic collaterals.

Another clinical feature of cirrhotic patients is hypogonadism manifested with impotence and small testes in males and dysmenorrhea, infertility and benign cysts of the breast in females. Feminisation of male patients occurs characterised by gynaecomastia and loss of body hair. Finally parotid enlargement is frequent in patients with cirrhosis

1.11 Complications of cirrhosis

The main complications of cirrhosis result from portal hypertension , portasystemic shunting and liver failure and include variceal haemorrhage, hepatic encephalopathy, ascites, renal dysfunction (hepatorenal syndrome) and pulmonary dysfunction (hepatopulmonary syndrome). We will examine in more details the pathogenesis of hepatic encephalopathy.

1.12 Chronic Hepatic encephalopathy

Chronic hepatic encephalopathy (CHE) is a complex neuropsychiatric syndrome that occurs in patients with cirrhosis and has a potential for full reversibility. It is characterised by global depression of the central nervous system and has different degrees of severity (Table 1.2). In the majority of patients CHE is subclinical and no overt signs and symptoms are present. Those patients have altered reaction times and impaired ability to perform psychometric tests. In 30% of patients the full blown syndrome is present with alterations in behaviour, mood, personality and disturbances

in consciousness. CHE is usually episodic and relapsing but some patients exhibit a chronic protracted course (Jalan et al, 1996).

Table 1.2

West Haven criteria for grading mental state in hepatic encephalopathy

Grade 0	No abnormality detected clinically
Grade 1	Trivial lack of awareness, euphoria, anxiety
	Shortened attention span
	Impaired performance in addition or substraction
Grade 2	Lethargy, apathy, disorientation for time and space
	Obvious personality change
	Inappropriate behaviour
Grade 3	Somnolence to semi-stupor but responsive to stimuli
	Confusion
	Gross disorientation.
Grade 4	Coma
	Mental state not testable

1.12.1 Pathogenesis

Many theories regarding the pathogenesis of CHE were popular at different times but the exact mechanisms remain unclear. We are going to summarise the most important elements that are thought to play a role in the pathogenesis of this syndrome.

1.12.2 Alterations in the blood-brain barrier

Patients with CHE exhibit a functional derangement of this protective mechanism (Kato et al, 1992). There is an increase in the transport of neutral amino acids and a decrease in the transport of basic amino acids (Ono et al, 1978). This could be fundamental in the development of CHE because levels of amino acid neurotransmitters in the brain, such as glutamate, aspartate and glycine are directly affected and the increased concentration of aromatic amino acids transported across the blood brain barrier act as substrates for the synthesis of neuroactive substances such as serotonin, kynurenine, quinolinic acid and tryptamine (Fischer et al, 1971). Although the mechanism of this derangement is not clear, it is thought that the disturbance of the blood brain barrier provides the mechanism by which other factors in CHE may operate.

Changes in energy metabolism occur in the brain in patients with CHE. There is decreased cerebral blood flow and decreased consumption of glucose and oxygen. There seems to be a derangement in high energy phosphate bonds as well (Lockwood et al, 1987). Although energy metabolism in the brain seems to be deranged, there is so far no direct evidence linking the energy metabolism alterations to the cause of CHE. It is thought that they could be secondary epiphenomena.

1.12.3 Ammonia

Like other gut derived toxins ammonia fulfils the criteria for potential neurotoxins that contribute to CHE. All candidates should be produced in the gut, be nitrogenous, be found in the portal circulation, be metabolised by the normal liver and should cross the blood brain barrier (Basile et al, 1991). Ammonia is produced by the action of gut bacteria on food, by breaking down of dietary proteins, by metabolising glutamine and other circulating amino acids. It is detoxified in the liver through the urea cycle and through the synthesis of glutamine from glutamate in the liver, muscle and brain. In cirrhotics, due to dysfunction of the hepatocytes and portosystemic shunting, increased concentrations of ammonia in the blood and the CNS have been demonstrated (Lockwood et al, 1991).

Ammonia diffuses readily into the brain and can induce intracranial hypertension in animal models (Voorhies et al, 1983). The main pathologic change in the CNS associated with CHE is the development of Alzheimer's Type II astrocytes which are characterised by large, pale nuclei, large nucleoli, and an increased number of intracellular organelles that are involved in the production of metabolites by the cell (Adams et al, 1953). It is postulated that ammonia plays a pivotal role in that phenomenon. Ammonia in the brain is detoxified by combination with glutamate and production of glutamine. The enzyme responsible for this reaction in the brain is glutamine synthetase and is exclusively found in the astrocytes. Also, upregulation of glutamate transporter GLT-1 is found on cerebral astrocytes in animal models of CHE. Ammonia inhibits the channels that pump chloride ions into the cells. This leads

to increased intracellular concentration of chloride, and inhibition of excitatory postsynaptic electrical activity (Goldstein, 1984).

Ammonia can also facilitate the uptake of tryptophan in the brain. Tryptophan is a substrate for the generation of neuroinhibitors like serotonin and tryptamine which were found to be elevated in CHE and neuroexcitators like quinolinic acid which was also found to be elevated in CHE. Ammonia is also implicated in the neurodrepression of CHE via reducing glutamatergic neurotransmission.

Although ammonia seems to be a key element in the development of CHE its significance must be put into context. In favour of the ammonia hypothesis is the fact that hyperammonaemia in other conditions produces a CHE like picture, as well as the fact that treatment of patients with non-absorbable disaccharides reduces blood ammonia levels and improves encephalopathy. The hypothesis is also supported by the fact that feeding ammoniagenic substances to normal volunteers produces encephalopathy. However-and this contradicts the hypothesis- there is no correlation between ammonia levels and severity of encephalopathy and CHE can occur with normal ammonia levels (Jalan et al, 1996).

Based on the ammonia hypothesis various treatment regimes for CHE have been developed. Patients are given a low protein diet to minimise ammonia production, lactulose and lactitol that can reduce ammonia available for absorption by incorporating it into bacterial proteins. They are also prescribed non-absorbable antibiotics to reduce production of ammonia by gut flora. Another new treatment is the administration of sodium benzoate that facilitates hippurate synthesis from excess ammonia (Jalan et al, 1996).

1.12.4 Glutamate

Besides being an intracellular buffer in astrocytes for ammonia detoxification, glutamate is also the predominant excitatory neurotransmitter in the brain. Glutamate concentration is regulated at the synaptic level of the neuron. Astrocytic glutamate uptake is impaired in CHE (Raabe, 1987). Also glutamate binding sites in the postsynaptic neuron are down regulated (Norenberg et al, 1985). Both mechanisms contribute to increased extracellular concentrations of glutamate. This could be a contributing mechanism to the overall impaired brain function in CHE.

Based on the glutamate hypothesis, a new treatment has been developed recently to treat exacerbations of CHE. L-Ornithine-L-Aspartate (LOLA) a dicarboxylate that promotes glutamine synthesis in the hepatocytes and stimulates the urea cycle has been used in clinical trials with satisfactory results (Lewelling et al, 1991).

1.12.5 GABA and endogenous benzodiazepines.

Another theory that was en vogue during the early 90's was the one involving ?-aminobutyric acid (GABA), and endogenous benzodiazepines. GABA is the major inhibitory neurotransmitter in the brain. It is a product of glutamate in the brain and exerts its action in the brain through $GABA_A$ receptors (Jalan et al, 1996). The activity of these receptors is thought to be regulated by endogenous benzodiazepines that bind to a closely linked site (Jones et al, 1988). Endogenous benzodiazepines are increased in the blood and brain of CHE patients (Olasmaa et al, 1990). The evidence of deranged GABA metabolism is not convincing. Flumazenil, a

benzodiazepine antagonist has been used in the treatment of CHE with disappointing results (Pommier-Layrargues et al, 1994).

1.12.6 False neurotransmitters.

Noradrenaline, dopamine and serotonin are cerebral neurotransmitters. Dopamine and noradrenaline seem to be decreased in the brain in CHE whereas serotonin seems to be increased in the brain in CHE. The net result could therefore be an increase in neuroinhibition (Jalan et al, 1996). Aromatic amino acids in the brain in CHE are increased and can block the enzyme tyrosine hydroxylase which is vital for the production of cathecholamines. However production of other amines such as tyramine and octopamine is not affected. Those false neurotransmitters could bind to catecholamine receptors, induce neuroinhibition and further suppression of CNS function (Fischer et al, 1971).

Based on that theory, branched chain amino acids who compete for absorption sites with aromatic amino acids have been infused to patients suffering from CHE. The results so far have been disappointing (Morgan,1990) .

1.12.7 Manganese

Theories have been developed for a role of manganese in CHE. They are based on the observation that similar patterns of hyperintesity in the basal ganglia are observed in patients with chronic liver disease and manganese toxicity using T_1-weighed Magnetic Resonance Imaging (MRI) (Krieger et al, 1995). Increased manganese concentrations have been observed in the globus pallidus of patients (Pommier-Layrargues et al, 1995). Blood manganese concentrations have also been reported to

be increased (Krieger et al, 1995). It has been suggested that increase manganese concentration in the basal ganglia could be responsible for the extrapyramidal features of patients with CHE but this has not been supported overwhelmingly.

As this brief synopsis has tried to show there are many theories regarding the pathogenesis of CHE and many substances have been implicated in it. The evidence does not support the idea of one substance being only and wholly responsible for the pathogenesis of CHE and it seems that CHE is a multifactorial disease and there is scope for further studies and investigation into the role of other substances in its pathogenesis.

1.13 Fulminant Hepatic Failure

Fulminant hepatic failure (FHF) is a clinical syndrome characterised by massive hepatocellular dysfunction, in the absence of chronic liver disease, resulting in hepatic encephalopathy. The failing liver has a potential to regeneration can recovery can occur without permanent sequela.

1.13.1 Definition

The definition of fulminant hepatic failure is still cause for debate. In the original definition by Trey and Davidson (1970), encephalopthy has to develop within 8 weeks of the onset of symptoms for the syndrome to be called fulminant hepatic failure. If encephalopathy presents between 8 and 24 weeks then the syndrome is defined as late-onset hepatic failure. Various other definitions have been brought forward during the last thirty years. Recently, the King's College Hospital Group in

London, proposed a new terminology with the term acute liver failure at its centre. Patients presenting within 7 days of the onset of symptoms were termed hyper-acute liver failure, those presenting within 8 -28 days were termed acute liver failure and those presenting between 29 and 72 days were termed subacute liver failure (O'Grady et al, 1993). Irrespective of terminology, all studies seem however to agree that the patients with the most rapid onset of encephalopathy have the best chance of spontaneous recovery. We have adopted the term fulminant hepatic failure throughout this thesis to describe the acute liver injury and failure syndrome.

1.13.2 Prognosis and aetiology

Fulminant hepatic failure is fairly uncommon and has a high mortality approaching 80% in some series (O'Grady et al, 1993). The prognosis depends on aetiology and concomitant liver disease.

Paracetamol overdose which is the most frequent cause of FHF in the UK has a rather better prognosis with an overall mortality of around 40% (Plevris et al, 1998). Paracetamol metabolism produces an unstable nucleophilic intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI), which damages cells, if produced in amounts greater than can be detoxified by hepatic protective substances such as glutathione. Paracetamol hepatotoxicity is more common in alcoholics and in patients taking enzyme-inducing drugs that induce the cytochrome P_{450} system, which produces the toxic metabolite. The antidote N-acetylcysteine (NAC), by acting as a sulfydryl donor neutralises the toxic compound. There is evidence to suggest that although patients

benefit most within 10hrs of the overdose from NAC, late administration is also beneficial (Harrison et al, 1990).

Hepatitis A is the commonest cause world wide. It has also overall a better prognosis with survival rates of around 50% although only 0.4% of patients suffering from acute hepatitis A will develop a fulminant course (Hoofnagle et al, 1995). Although Hepatitis A virus is directly cytotoxic there is also evidence of cell mediated toxicity in fulminant hepatitis A.

Hepatitis B virus infection is a common cause of FHF in many countries. It is associated with HBV reactivation, HBe antigen/antibody seroconversion, coinfection and superinfection with Hepatitis D virus and HBV precore mutant infection. Other hepatotropic viruses like Hepatitis C Virus, Hepatitis E virus, Cytomegalovirus, Epstein-Barr virus, Herpes Simplex virus, Varicella-Zoster and Herpes virus-6 are all rare causes of fulminant hepatic failure (Tibbs et al, 1995). Other drugs can cause liver damage and most produce their effect on an idiosyncratic basis. Wilson's disease can produce a fulminant picture and Reye's syndrome is also characterised by encephalopathy, cerebral oedema and fatty liver (Plevris et al, 1998).

1.13.3 Pathology

The commonest histological finding in FHF, irrespective of aetiology is severe hepatocyte necrosis. In paracetamol overdose a panacinar eosinophilic necrosis is observed 2-3 days after the injury (Portmann et al, 1975). The centrizonal areas are mostly affected, while the periportal areas are commonly preserved. Cholangioles are proliferating in the damaged areas. Cholestasis is observed in the remaining hepatocytes and is most prominent after sepsis. Inflammation is often

lacking until the late stages of the disease. Fatty changes in the hepatocytes are observed in some causes of FHF. The remaining liver cells have the ability to regenerate and this has been demonstrated by immunostaining of proliferating cell nuclear antigen (Koukoulis et al, 1992).

1.14 Clinical course of acute liver failure

The clinical course is that of a progressive multi organ failure. The loss of functioning hepatocytes has adverse metabolic consequences. The toxins produced by the liver have systemic effects and the immune system is compromised. We will briefly describe the multi organ failure concentrating more on the brain, the renal and cardiovascular manifestations and the coagulopathy.

1.14.1 Cerebral dysfunction

Encephalopathy is the hall mark of FHF. The West Haven criteria were produced to assess the different degrees of severity as in CHE. As patients progress to grade III or IV coma, cerebral oedema which is a unique manifestation of fulminant hepatic failure appears. Cerebral oedema occurs in 70-80% of FHF patients in coma (Lee,1993). The pathogenesis of cerebral oedema is not clearly understood. The most plausible hypothesis implicates ammonia and glutamate. It is thought that glutamate in the astrocytes of the brain detoxifies ammonia by combining with it and producing glutamine. The glutamate-glutamine system serves according to this hypothesis as a buffer system in the astrocytes. Myo-inositol, another astrocytic compound is thought to buffer the intracellular water content of the astrocytes.

Excess ammonia exhausts the glutamate-glutamine system particularly as uptake of extracellular glutamate is impaired in the failing astrocytes. An excess of intracellular ammonia would also deplete the myo-inositol buffering system and the net result would be astrocytic swelling in the brain (Goldstein, 1984). Although this theory has generated a lot of enthusiasm, it remains to be adequately proven in the context of FHF.

Cerebral oedema is the primary cause of death in most FHF patients. Intracranial pressure (ICP) rises and the clinical signs of it are summarised in Table 1.3. It is also accompanied by reduced cerebral blood flow and reduced cerebral oxygen consumption. This series of events leads to cerebral herniation and coning through the foramen magnum and eventually the patient develops apnoea and dies. The clinical signs can occur late or be absent and intensive monitoring can be potentially life saving. Intracranial pressure monitoring with an epidural bolt can show early ICP rising and appropriate therapy with mannitol or thiopentone can be instituted (Blei, 1991). Oxygen delivery to the brain can be monitored via a jugular bulb catheter, but this is an invasive and potentially dangerous procedure.

Table 1.3

Clinical signs of raised intracranial pressure

1 Systolic hypertension (paroxysmal or sustained)

2 Bradycardia

3 Increased muscle tone, opisthotonos, decerebrated posturing

4 Pupillary abnormalities (sluggish or absent response to light)

5 Brain stem respiratory patterns, apnoea

1.14.2 Infection and sepsis

FHF patients are prone to develop infections and generalised sepsis because their immune system is compromised. Complement concentrations, bacterial opsonisation, white cell chemoattraction and bacterial killing and Kupffer cell function are all impaired. Patients are also subject to an array of invasive procedures (Canalese et al, 1982). Bacterial and fungal infections complicate FHF. The commonest bacteria are Gram-positive bacteria especially Staphylococcus aureus. The commonest Gram-negative organism is Escherichia coli. Fungal infections are particularly dangerous and mainly due to Candida albicans. Typical signs of infection are absent in those patients. Early use of broad spectrum antibiotics is recommended.

1.14.3 Haemodynamic abnormalities

FHF is associated with increased cardiac output and reduced peripheral vascular resistance both systemic and pulmonary (Bihari et al,1985). Clinically patients are profoundly hypotensive. Oxygen delivery to the tissues increases but the oxygen extraction ratio and the oxygen consumption decrease, leading to impaired tissue perfusion, which is the cause of increased lactate production from the hypoxic cells.

Treatment is by fluid replacement initially and if that proves ineffective then vasopressive agents such as epinephrine and norepinephrine.

1.14.4 Coagulation abnormalities

Cogulopathy in FHF is the result of inadequate synthesis and increased consumption of clotting factors and platelet abnormalities. Monitoring of the coagulopathy through prothrombin time and International Normalised Ratio (INR) is the best indicator of changing liver function. Severe haemorrhage is rare, so prophylactic correction of coagulopathy is contraindicated.

1.14.5 Respiratory complications

They are the result of central hyperventilation and respiratory alkalosis as the patient becomes progressively encephalopathic. When cerebral oedema develops, worsening hyperventilation may herald sudden apnoea and tentorial herniation. Endotracheal intubation is usually undertaken in patients in grade III or IV coma and mechanical ventilation is instituted. This also helps to prevent aspiration of gastric contents and pulmonary infection (Plevris et al, 1998).

1.14.6 Renal failure

An oliguric renal failure develops in half of all FHF patients. Acute tubular necrosis and functional renal failure may be found. Renal blood flow is reduced and renin and angiotensin are increased (Wilkinson et al, 1977). Renal failure is treated with either continuous venovenous or high flow haemofiltration as solute and osmolality changes should be slow to prevent development of cerebral oedema (Plevris et al, 1998).

1.14.7 Electrolyte and metabolic abnormalities

Hyponatraemia commonly develops as sodium is shifted intracellularly and the Na^+/K^+ -ATPase is inhibited. Hypokalaemia and hypophosphataemia can also occur (Wilkinson et al,1974). Hypoglycaemia is a hall mark of FHF as the liver has depleted glucagon stores and cannot clear insulin properly. Routinely patients with FHF are given 10% glucose solutions intravenously.

1.15 Treatment of acute liver failure.

Orthotopic liver transplantation is the most effective means of therapy for patients with FHF whose prognosis is poor. The results for liver transplantation for FHF have significantly improved during the last few years and 1-year survival ranges between 50% and 80% in different centres (McCashland et al,1996). What has emerged from studies in Europe and the U.S. is that overall success rates are lower in the setting of FHF than in elective orthotopic liver transplantation (Bismuth et al, 1996). This can be attributed to the poor clinical condition of the patients at the time

of transplantation, but also to the inferior quality organs accepted due to the critical condition of the patients.

Patients for transplantation are selected based on the King's College Hospital criteria (Table 1.4). Those criteria were found to have a good predictive accuracy as to which patients have a very poor prognosis if managed conservatively but can be fulfilled days or weeks after the onset of disease (O'Grady et al, 1989). Although orthotopic liver transplantation is the only hope of survival for these patients, only a selected few can benefit from such therapy.

In this thesis by using ^{1}H NMR Spectroscopy, in association with biochemical data we have established criteria that could predict early poor outcome of patients with acute liver failure both in the paracetamol overdose group and in the non paracetamol overdose group so that more time can be available in the search for a suitable donor organ.

Many patients with acute liver failure will never be transplanted anyway as they deteriorate too quickly, have contraindications to transplantation or the shortage of organ donors implies that an organ can not be found quickly enough.

Table 1.4

King's College Hospital criteria for liver transplantation in acute liver failure

Paracetamol

pH of arterial blood<7.30 (irrespective of the grade of encephalopathy)

or

Prothrombin time > 100 sec and serum creatinine > 300 μmols/L in patients with grade III or IV encepahlopathy

Non-paracetamol patients

Prothrombin time > 100 sec (irrespective of the grade of encephalopathy)

or

Any three of the following variables (irrespective of the grade of encephalopathy)

Age < 10 or > 40 yr

Aetiology- Non A Non B hepatitis, halothane hepatitis, idiosyncratic drug reaction

Duration of jaundice before the onset of encephalopathy > 7 days

Prothrombin time > 50 s

Serum bilirubin > 300 μmols /L

To overcome these difficulties alternatives to whole organ transplantation are sought. Auxilliary transplantation is one of them. A partial liver graft is placed either beside the native liver (heterotopic) or in place of a removed liver lobe (orthotopic). Although initial results are encouraging it is a technically

demanding operation which has a high postoperative rate of complications (Chenard-Neu et al, 1996).

Another alternative in the attempt to bring a stable patient to transplantation is the total removal of the failing liver or its devascularisation while the patient awaits for donor organ to become available. There is evidence that this corrects the cerebral oedema and the haemodynamic abnormalities (Ringe et al, 1993). Initial evidence suggests that this could be of benefit to increasingly unstable patients for whom there is a high probability for a graft to become available (Plevris et al, 1998).

Liver transplantation in the FHF setting faces the problem of organ donor shortage. As the native liver has a potential for full recovery a system that would provide temporary support for the failing liver in order to allow for regeneration of the native liver would be ideal (Wang et al,1995). Another interesting theory for temporary liver support is hepatocyte transplantation of cultured hepatocytes into the native liver or spleen where they take temporarily over the function of the native liver until this recovers and the transplanted cells are rejected (Demetriou et al, 1998).

Both those interesting liver support modalities require the availability of primary porcine hepatocytes on demand as they are mostly used in emergency circumstances. Primary porcine hepatocytes are thought to be the ideal cell for use in those modalities. Their optimal culture and preservation conditions though remain unclear. Those cells should be able to exhibit an active metabolism in culture before they are used in liver support modalities.

A lot of work has been recently undertaken in our Liver Cell Biology Laboratory in order to understand the pathophysiology of acute liver failure and to

develop therapeutic modalities for this syndrome. This thesis is part of the collective effort of our laboratory to better understand the physiology and pathophysiology of the liver.

CHAPTER 2

EXPERIMENTAL METHODOLOGY.

2.1 Cell culture supernatant.

Experiments in cell culture supernatants and plasma samples were performed using the proton nucleus as the observed nucleus. We have opted for ^{1}H NMR spectroscopy as it enabled us to have detailed fingerprints of low molecular weight substances in the biofluids investigated. Acquiring and editing a ^{1}H spectrum was relatively easy and quick and most of the necessary information about amino acids and intermediates of metabolic pathways and their concentrations was present. Our spectrometer, a Varian INOVA 600 MHz instrument, gave us enough information in1D and 2D spectra to enable us to assign and quantitate most of the important metabolic intermediates.

Specific problems related to the physicochemical properties of the biofluids investigated were addressed and tackled in this thesis.

2.1.1 Effect of sample temperature.

Although changes in temperature will have an effect on the sharpening of signals by methylene and methyl groups (Otvos et al, 1987) this is most prominent when those peaks are part of a complex macromolecule such as lipoproteins. Using the peak of the singlet methyl resonance of a known amount of Sodium 3-(trimethylsilyl 2,2,3,3-$^{2}H_4$) -1 propionate (TSP) at zero ppm as the only variable we

investigated the effect of temperature changes in peak intensity. Temperature ranged from 285^0 K to 317^0 K. The signal intensity did not change significantly between the two extremes and we chose the ambient temperature of 298^0 K as the temperature for our experiments.

2.1.2 Effect of sample pH.

Samples of plasma have a very constant pH that varies between 7.20 and 7.50 at the maximum. Storage of samples at - 40^0 C did not influence their pH after thawing. Although previous studies have postulated that aromatic amino acids are not NMR visible in neutral pH conditions (Gartland PhD thesis, 1988), our experiments did not confirm that and the peaks from histidine, tyrosine and phenylalalnine were clearly visible and easily assigned to the relevant amino acids. Their concentrations in control plasma were the same as the ones obtained by reverse phase High Pressure Liquid Chromatography (HPLC) performed in our laboratory. This was not true for citrate as its high affinity to albumin and the binding that results, made citrate resonances NMR invisible in neutral pH. As titration of samples to pH < 2 for identification of citrate was time consuming it was thought that not enough information would be gained to warranty acidification of all samples and all experiments were performed in neutral pH. Sample pH in the culture supernatant samples varied between 4.50 and 7.5. As albumin binding of low molecular weight substances was not an issue, the only problem we faced was the possibility of peaks shifting with changes in pH. But as the substances were few and the shifting was not pronounced, there was no signal overlapping due to changes in pH and the intensity of

the peaks did not change. As a result the experiments were conducted without pH titration.

2.1.3 Effect of storage and freezing of samples.

There were concerns that freezing of samples might affect the concentrations of the substances and that storage time might have similar results (Nicholson et al, 1989). All samples were analysed less than six months after acquisition. Both culture supernatants and plasma samples were analysed fresh and then after six months of freezing. The results showed that the storage conditions (i.e. freezing at -40^0 C) did not adversely affect the samples and the concentrations of low molecular weight substances studied were the same.

2.1.4 Effect of anticoagulants and chelating agents.

Plasma is a constituent of blood which is separated from blood after an addition of an anticoagulant. The most widely used anticoagulants are trisodium EDTA and Lithium Heparin. EDTA can also be used as a chelating agent that is able to form complexes with metals present in plasma such as calcium and magnesium. This would have a beneficial effect also in the intensities and sharpness of peaks from substances that would form complexes with those metals and become NMR invisible (Nicholson et al 1983). This is mostly true for citrate which was not studied in our experiments. As EDTA peaks overlap to an extensive amount to the peaks of glucose and methylamines it was thought best to collect samples on Lithium Heparin even though we were adding another metal that could form complexes with low molecular weight substances.

2.1.5 Effect of addition of D_2O as lock.

D_2O is an obligatory addition to all aqueous samples as it provides an internal lock field for the spectrometer. Literature at present would suggest that addition of D_2O in excess in a sample would result in some resonances being lost. In particular, the resonances from histidine, acetoacetate and other ketone bodies can be affected (Lindon et al. 1999). We have experimented with different amounts of D_2O and we observed this phenomenon. For our experiments we had to introduce a minimum amount of lock so as not to disturb the intensities of the above mentioned substances. We routinely used 75 µl of D_2O as a field lock. This resulted in some loss of resolution in very low concentrations.

2.1.6 Effect of albumin -ligand binding in plasma samples.

Many plasma components can exist either free in solution or bound electrostatically to albumin. This is true for lactate, acetate, tyrosine, tryptophan, phenylalanine and citrate (Nicholson et al. 1993). Bell et al (1989) have shown that almost 30% of lactate becomes NMR invisible when bound to albumin under physiological conditions. The addition of ammonium chloride was reported to turn lactate NMR detectable. We were able to confirm that and in addition there was significant increase in intensity in the peaks of acetate as well. No changes were seen in the peaks of tyrosine and phenylalanine. Their concentrations were similar to concentrations obtained by HPLC methods in our laboratory and to published references for control samples. The addition of ammonium chloride did not have any effect on tryptophan that remained NMR invisible in plasma. Ammonium chloride

was added routinely in our samples as a solution of 0.5 mmols/L and the added volume was 100µl.

2.2 NMR Spectroscopy monitoring

The concentration of the following substances in the culture supernatants was measured, using NMR spectroscopy: Acetate, acetoacetate and ß-hydroxybutyrate as those ketone bodies are mainly exported by liver cells (Fuchs et al, 1994; Duee et al, 1994; Gerlach et al, 1996), leucine, isoleucine, phenylalanine and tyrosine as the key ketogenic amino acids used by the hepatocyte (Gerlach et al, 1996), glucose, alanine, glycine and threonine concentration as a measure of gluconeogenesis and to monitor the cells energy requirements (Seglen 1974; Seifter et al, 1994), lactate and pyruvate as a measure of the active aerobic and anaerobic glycolysis (Seglen, 1974), valine, isoleucine and methionine concentrations as succinate formation precursors and possible substrates for the Kreb's cycle (Kinsell et al, 1948; De Blaauw et al, 1998) and the concentrations of glutamate, glutamine histidine and arginine as indices of active transamination and urea synthesis (Lang et al, 1990; De Blauw et al, 1998). Ethanol found within supernatants was also measured. Glycerol, myo-inositol, methylamine, dimethylamine and TMAO concentrations were measured in plasma samples.

2.3 Sample preparation for NMR spectroscopy.

Samples were prepared by adding a D_2O solution of (75 µl) to culture supernatants (700 µl) or to plasma samples of the same volume providing an internal field frequency lock for the spectrometer. Chemical shifts were referenced internally

to the singlet methyl resonance of Sodium 3-(trimethylsilyl 2,2,3,3-$^{2}H_4$) -1 propionate (TSP) at zero ppm. We used TSP as an internal reference standard; it is less toxic and cheaper than formate which some studies suggest as the ideal internal standard. Furthermore, as the laboratory had significantly more experience with TSP and most papers in the literature have used TSP we opted for TSP throughout our experiments

2.4 Proton NMR spectroscopy.

2.4.1 Presaturation technique.

^{1}H- NMR spectra were measured on control William's E medium, and supernatants of culture media. Data were acquired on a Varian INOVA 600 NMR Spectrometer operating at 600 MHz for protons. All spectra were acquired at ambient probe temperature (298 $\pm$ 0.2 ^{o}K). For each sample 128 transients (FID's) were acquired into 32K complex data points, over a spectral width of 6 KHz. 30^{0} pulses were applied with an acquisition time of 4.0 sec, followed by a recovery delay of 8 s, to allow for complete relaxation and recovery of the magnetisation. Water signal suppression was achieved by applying a gated secondary irradiation field at the water resonance frequency during the recovery delay. Spectral assignments were made by reference to literature values of chemical shifts in various media and biological fluids (Nicholson et al, 1989, Lindon et al, 1999) and coupling constants.

2.4.2 CPMG sequence.

The CPMG sequence was used in samples with a plethora of macromolecules in solution. This was valid for plasma samples that contained albumin and macromolecules. The CPMG sequence has short spin-spin relaxation times and attenuates protein resonances enough for low molecular weight substances to be measured without interference.

90^0 - τ - 180^0 - τ (1^{st} echo) - τ -180^0 - τ (2^{nd} echo) .....

For each sample 256 repeats of the CPMG sequence were acquired into 32K complex data points over a spectral width of 6 KHz. 90^0 pulses were applied with an acquisition time of 3.0 s, followed by a recovery delay of 15 s, to allow for complete relaxation and recovery of the magnetisation. CPMG was used to enable us to identify low molecular weight substances in small concentrations but for the integration process the simple presaturation spectra were used.

The use of the CPMG sequence in plasma samples has some drawbacks that could jeopardise the validity of the measurements. All observed nuclei should be equivalent and the spin-spin coupling observed results in a broadening of the lines studied. We have tried to include as much area under the line as possible by integrating on the whole line width. As some of the small molecules observed had big $\mathbf{T_1}$ relaxation times the recovery period between pulses was adequate for all molecules to relax in order to avoid selective saturation of the peaks of those molecules. The real recovery period in our CPMG experiment was 15 seconds that is 5 times the $\mathbf{T_1}$ relaxation time of the pyruvate molecule.

2.4.3 J-resolved experiments.

We applied J-resolved experiments to simplify the plasma spectrum and to enable us to resolve the overlap resonances in the most crowded region of d 3- 4 ppms. It was used in conjunction with COSY experiments to identify overlapping resonances in a few selected plasma samples.

2.4.4 ^{1}H-^{1}H coupling homonuclear COSY experiments .

COSY provided confirmation of the presence of a number of amino acid resonances in particular branch chain amino acids, threonine and glutamate - glutamine. Other small molecules are resolved and also polyols as myo - inositol and connectivities from many lipidic resonances can be observed. COSY was also performed on selected few plasma samples.

Quantitation of compounds present in the culture supernatants was achieved by two alternative means. (a) Integrals were measured relative to that of a known quantity of TSP (10 mg/ml) present as an internal standard to the solution. (b) When signals were partially overlapped, peak height measurements were used, taking into account the appropriate coupling pattern of intensities of the non-overlapped lines. The computer program used for the integrations was the VARIAN 6.1 in a SOLARIS environment on a SUN 3.0 data station.

2.5 Reproducibility and variability of NMR experiments.

In order for our method to be valid it had to be reproducible with an acceptable interassay and intraassay variability. We have compared our results to two different standards. In the context of cell culture supernatants we have compared the

concentrations of low molecular weight substances to those in the manufacturer's product book. We have also compared our results with results on amino acid concentrations by HPLC. Table 2.1 shows the concentrations of the different substances present in William's E medium as determined by High performance liquid Chromatography (HPLC) and as determined by our experimental ^{1}H NMR methods. Coefficients of variation were determined for the two methods for all the substances stated in table 2.1. They were similar for both methods. Inter-assay variability was found to be lass than 10% for all the substances measured. The intra-assay variability of our NMR method was tested and was found to be less than 5%.

TABLE 2.1

Comparison of concentrations of substances present in Wiiliam's E medium as measured by HPLC and NMR spectroscopy

	BIOCHEMISTRY	NMR
LEUCINE	**0.4 ± 0.02**	**0.44 ± 0.04**
ISOLEUCINE	**0.27 ± 0.02**	**0.28 ± 0.01**
VALINE	**0.30 ± 0.03**	**0.31 ± 0.03**
THREONINE	**0.23 ± 0.02**	**0.21 ± 0.02**
LACTATE	**0**	**0**
ALANINE	**0.41 ± 0.02**	**0.43 ± 0.03**
GLUTAMINE	**1.41 ± 0.03**	**1.38 ± 0.04**
GLUTAMATE	**0.21 ± 0.02**	**0.21 ± 0.02**
METHIONINE	**0.07 ± 0.005**	**0.07 ± 0.005**
PYRUVATE	**0.20 ± 0.03**	**0.18 ± 0.03**
GLUCOSE	**7.70 ± 0.45**	**7.93 ± 0.34**
TYROSINE	**0.19 ± 0.02**	**0.18 ± 0.02**
HISTIDINE	**0.07 ± 0.01**	**0.06 ± 0.01**
PHENYLALANIINE	**0.14 ± 0.02**	**0.13 ± 0.02**
ACETATE	**0**	**0**
ETHANOL	**0**	**0**

For the plasma samples we tested the concentrations of glucose, creatinine and lactate against standard automated assays. The inter-assay variabilty for all measurements using coefficients of variation was less than 10%.

2.6 Intracellular vs extracellular measurements.

We should be aware of the fact that we tested supernatants from cell cultures and we calculated the concentrations of metabolites in key intracellular pathways. The question that we had to address is whether cell extracts and measurements of intracelluar metabolites would provide a more accurate reflection of the processes studied.

Intracellular concentrations of key metabolites are much smaller than milieu concentrations. To get spectra that would have peaks above the detection limit for the substances studied we had to sacrifice 80 million cells every time we were to sample our cultures. This was not practical and even then the spectra showed predominant features of glucose and citrate spectra whose concentrations intracellularly are much higher than those of amino acids. In addition to that hepatocytes have always been thought of as the factory of the organism where although everything is assembled and produced , it is then exported in great quantities to other organs where substances are used or excreted. Ketone bodies are exported to muscle and kidneys, lactate is excreted whenever possible, urea and by products of nitrogen and ammonia elimination are excreted in the milieu and amino acids are constantly remodelled to form proteins or to fuel energy providing pathways. It was thus thought that differences in concentrations of substances in the supernatant of the

culture would more accurately reflect the overall metabolic status of the cells over a period of time.

To study the concentrations of intracellular contents of the cells it was necessary to freeze dry the cell extracts and to reconstitute them in a smaller volume. This eliminated volatile substances like the ketone bodies and would result in break down of the energy rich phosphate bonds in the cell. Contamination with membranes particles is almost inevitable and membrane lipoproteins made assignments for substances even more difficult. In addition to that reconstitution used D_2O as solvent and as we showed earlier this resulted in distorsion of peaks and loss of signal intensities. At the end we opted for the study of cell culture supernatants that would provide us with a picture of cell metabolism over a period of time , quickly and with no added cost

CHAPTER 3

STUDIES ON FRESHLY ISOLATED HEPATOCYTES

3.1 INTRODUCTION

Fulminant hepatic failure is frequently fatal. The reason for the high mortality of this disease lies primarily with its fulminant course, and irreversible destruction on the patient's native liver. Currently, the best treatment option currently is orthotopic liver transplantation (OLT). Although OLT is highly successful due to a shortage of organ donors, less than 30 % of patients with fulminant hepatic failure will benefit from it (Plevris et al.1998). This is the main reason for the current rush to develop alternatives to transplantation in those patients. Bioartificial liver support systems (BALSS) have been discussed previously. Many centres are currently developing BALSS and the preliminary results have been encouraging (Watanabe et al, 1997; Flendrig et al, 1997; Samuel, 1998). The key component of these devices is a substratum of cultured hepatocytes that would temporarily and intermittently replace the functions of the failing native liver. The choice of hepatocytes is not obvious. Human hepatocytes are so scarce that it would be unthinkable to develop a system based on those cells. Immortalised cell lines on the other hand offer an unlimited cell supply but there are problems with tumourigenicity and dedifferentiation (Newsome et al, 2000). Mammalian hepatocytes would be a more obvious source. Primary porcine hepatocytes have been regarded as the cells of choice as they can be readily obtainable with a sufficient yield and viability. They maintain differentiated functions in culture

and have high intrinsic metabolic activity (Naik et al, 1997; Donato et al, 1999).

Porcine hepatocytes cultured in William's E medium with the addition of 10% serum on various substrata and uncoated tissue culture plates showed no differences between them in differentiated metabolic functions suggesting that substrata are not necessary to culture PPHs (te Velde et al. 1995). However, culture of PPHs in media supplemented with serum might be ill- advised if the cells are to be used in BALSS connected to patients. Serum supplementation would make the transmission of diseases more probable.

In our tissue culture laboratory we have cultured our cells on biomatrix-free tissue culture plates, in serum free, chemically defined media containing highly purified recombinant growth factor, insulin and dexamethasone. Insulin was used to enhance initial cell attachment (Gerlach et al, 1994). Long epidermal growth factor stimulates DNS synthesis, cell growth, amino acid transport and protein synthesis (Michalopoulos et al, 1990). All three hormone contribute to the maintenance of differentiation and albumin synthesis. Dexamethasone also suppresses fibroblast growth (Sun et al, 1999)

Different culture media have been used in hepatocyte culture studies (Brodskii et al, 1989; Scott et al, 1991;Thompson et al, 1993; Coldham et al, 1998; Guzic et al, 1998,). We compared biochemical function and activity of PPHs in four commonly used culture media, William's E medium, medium 1640, Medium 199 and Hepatocyte Medium, to assess differences in the function of metabolic pathways of cultured PPHs between media and also at different time points of the cell culture experiments.

This study provides some insight into the metabolism of amino acids by hepatocytes in culture. The different amino acids provide substrata for the basic

metabolic functions of the cell. Some can only be diverted into one metabolic pathway while others can feed two or even three different metabolic pathways.

3.1.1 Metabolism of amino acids of interest

We are now going to look into the metabolism of the different amino acids in detail. Alanine, a key player in the hexose phosphate pathway, can be produced from threonine and can feed downstream the glucose pathway by transamination with a-ketoglutarate, producing pyruvate and glutamate. Pyruvate, alanine's α-ketoacid can either feed the Kreb's cycle through acetyl -CoA production or be transformed to lactate under anaerobic conditions.

Threonine that enters the cells can only be converted to alanine in order to be transformed to pyruvate. Other amino acids that can be converted to pyruvate include glycine, cysteine, serine and tryptophan.

Tyrosine can also feed the hexose phosphate pathway through production of oxaloacetate by transamination of a-ketoglutarate and production of phosphoenolepyruvate. The same applies to phenylalanine and aspartate. Phenylalanine can be converted to acetoacetate and acetyl CoA producing thus ketone bodies. Aspartate can also be produced from glutamate as substrate to feed the urea cycle.

Glutamate can either be converted to glutamine or feed the Kreb's cycle through a-ketoglutarate production or be produced from a-ketoglutarate by accepting an ammonium ion. Glutamine can only be produced from glutamate and is the metabolic end product of this pathway. Glutamine is used in DNA synthesis in cells undergoing mitotic division. Arginine and histidine can be transformed to glutamate

and that is their only breakdown pathway in the hepatocytes. Proline also needs to be transformed to glutamate, if it is to be effectively broken down by the hepatocytes. Acetyl -CoA can, as we mentioned, be produced from phenylalanine and tyrosine but also from isoleucine and leucine, lysine and tryptophan. This is the only breakdown pathway for leucine. Of course, in hepatocytes, the major pathway for acetyl -CoA production is fatty acid oxidation.

Some amino acids, namely isoleucine, valine and methionine can feed the Kreb's cycle via production of succinate. Production of succinate is the only pathway for oxidation of valine and methionine.

3.2 Materials and methods

3.2.1 Animals.

Weanling piglets (*Large white*: < 15 kg) were obtained from the Roslin Institute (Roslin, Scotland) on the day of isolation having been fed a standard diet and water *ad libitum*. All animals were treated in accordance with UK Home Office Guidelines (Scientific Procedures Act, 1986).

3.2.2 Chemicals

Type IV collagenase (from *Clostridium histoliticum*),EGTA, HEPES, albumin, Long Epidermal Growth Factor, all culture media Williams E, Hepatocyte medium, medium 199 and RPMI 1640 were from Sigma. Hank's balance salt solution (HBSS) buffer, PBS, L- Glutamine, Penicillin - Streptomycin and Amphotericin B were from ICN. All reagents were of cell culture grade. Other reagents were: Gentamicin (non- proprietary), Porcine Insulin (Pork Velosulin^{TM}, Novo Nordisk,

Denmark), Dexamethasone (David Bull Labs, England) and Aprotinin (Trasylol™, Bayer). All chemicals were of Analar grade.

3.2.3 Hepatocyte Isolation

Our method for isolating primary porcine hepatocytes has been described in detail elsewhere (Nelson et al, 2000). Briefly, piglets were sacrificed using an i.v. overdose of pentobarbitone sodium, the infrahepatic inferior vena cava was clamped and the suprahepatic inferior vena cava was cannulated. The liver was retrogradely perfused *in situ* with cold saline and excised. It was then transported to the laboratory on ice and perfused with prewarmed perfusion buffers in 5 steps. The liver was disrupted using collagenase, sequentially filtered in washing buffer, purified by sedimentation and centrifugation and the isolated liver cells suspended in Williams E medium. Cell viability was assessed using the trypan blue exclusion test using a Neubauer cytometer. Cell viability was assessed by percentage extracellular lactate dehydrogenase (LDH) activity using the COBAS FARA. Plating efficiency was assessed by measuring total protein using the COBAS FARA and comparing the results with baseline values.

3.2.4 Hepatocyte culture

Cells were seeded at a density of $8x10^6$ viable cells per 90mm plastic culture dish (Dow Corning, USA) in 8ml of each of the four test media (WE, HM, 199,1640) at 37^0 C. The culture media consisted of serum free hormonally defined media. The exact composition of each one of the four media can be found in Table 3.1. We used serum free media that were supplemented with the following substances: L-EGF 50

ng/ml, porcine insulin 10µg/ml, dexamethasone 1nmol/ml, glutamine 2µmols/ml, gentamicin 50mg/ml, penicillin 50mg/ml, streptomycin 10 mg/ml, and amphotericin B 2.5µg/ml. The culture dishes were shaken gently to aid cell dispersal and facilitate attachment, and then placed into a humidified incubator in an atmosphere of 95 % air and 5% CO_2. For the initial cell attachment phase the incubator was gassed with 30% O_2/5% CO_2 for 2 hours. On days 2 and 4 the culture dishes were aspirated and fresh media added.

3.2.5 NMR Spectroscopy

NMR Spectroscopy monitoring was performed as discussed in Chapter 2. No signals from acetoacetate, ß-hydroxybutyrate and glycine were identified and concentrations of those substances were consequently not measured. Sample preparation, data acquisition and quantitation of compound concentrations were performed as discussed in Chapter 2.

3.2.6 Statistical analysis

Comparisons between freshly isolated cells cultured in different media from the same isolation were made. The values of each experiment were the mean of eight cultures. Student's t-test was used to compare means of different groups. A p value of < 0.05 was taken as significant (two-tail test of significance). To compare between more than two groups we used the Tukey test based on the one factor independent measures ANOVA. Results are expressed in mmols/L and are corrected by the number of viable cells per dish.

TABLE 3.1

Concentrations of amino acids and keto acids in the four different media

	WE	1640	199	HM
LEU	0.4	0.27	0.64	0.67
ISOL	0.27	0.27	0.21	1.33
VAL	0.30	0.12	0.30	1.71
THRE	0.23	0.12	0.35	3.53
LACT	0	0	0	0
ALA	0.41	0	0.39	3.54
CITR	0	0	0	0
GLN	1.41	0.48	0.48	1.43
GLM	0.21	0.29	0.63	0.95
METH	0.07	0.07	0.14	0.70
PYRU	0.20	0	0	4.38
ASPR	0.14	0	0.28	0
GLU	7.70	7.70	3.88	0
TYR	0.19	0.20	0.22	1.16
HIST	0.07	0.07	0.10	1.13
PALA	0.14	0.08	0.21	1.06
ACET	0	0	0	0
ETH	0	0	0	0
GALA	0	0	0	3.5

3.3 RESULTS

Figure 3.1A shows a 600 MHz spectrum obtained from pure William's E medium and Figure 3.1B shows a spectrum from cell culture supernatant on day 2 from cultures in William's E Medium. Figure 3.2A shows a 600 MHz spectrum obtained from pure medium 1640 and Figure 3.2B shows a spectrum from cell culture supernatant on day 2 from cultures in Medium 1640. Figure 3.3A shows a 600 MHz spectrum obtained from pure medium 199 and Figure 3.3B shows a spectrum from cell culture supernatant on day 2 from cultures in Medium 199. Figure 3.4A shows a 600 MHz spectrum obtained from pure Hepatocyte Medium and Figure 3.4B shows a spectrum from cell culture supernatant on day 2 from cultures in HepatocyteMedium.

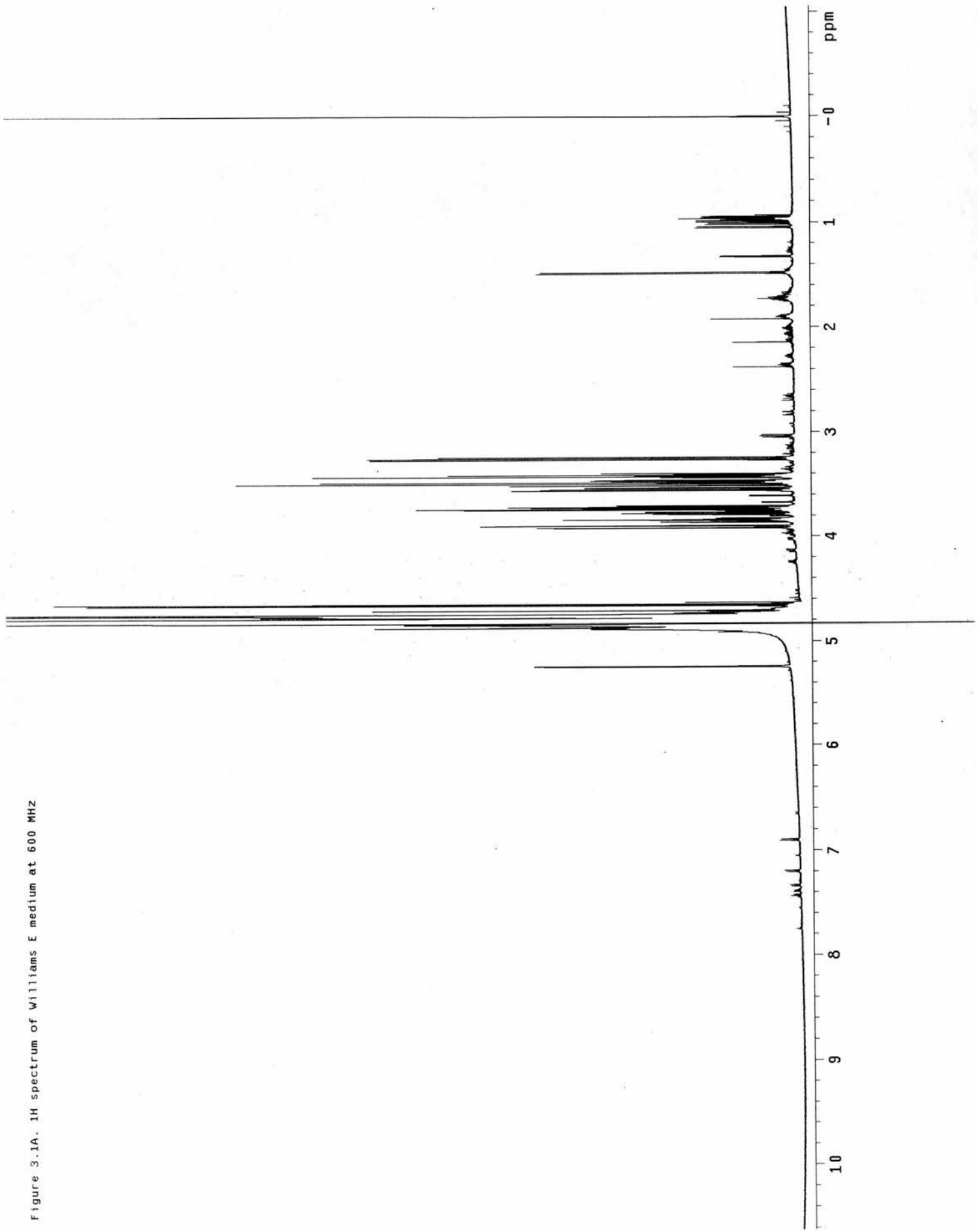


Figure 3.1A. 1H spectrum of Williams E medium at 600 MHz

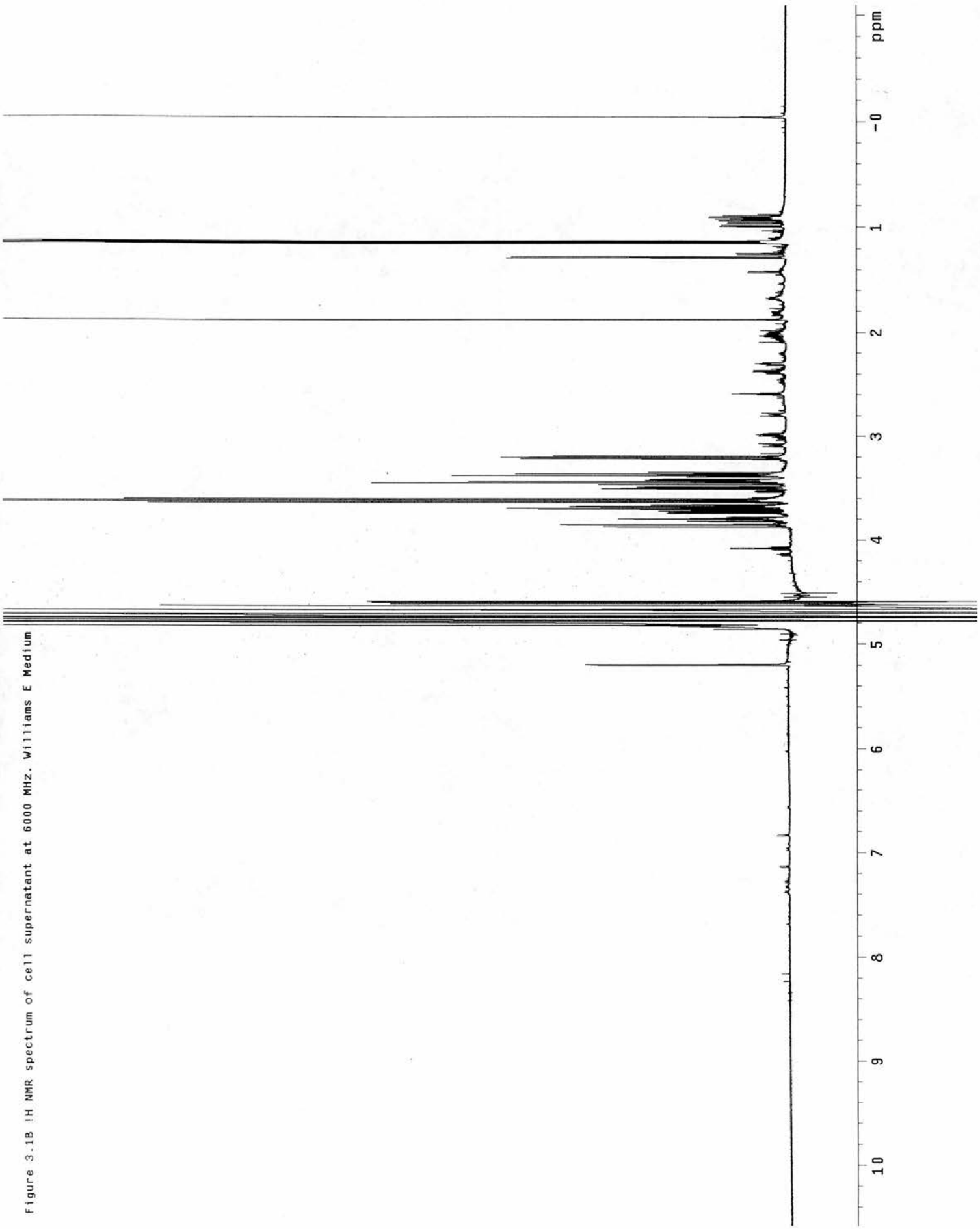


Figure 3.1B !H NMR spectrum of cell supernatant at 6000 MHz. Williams E Medium

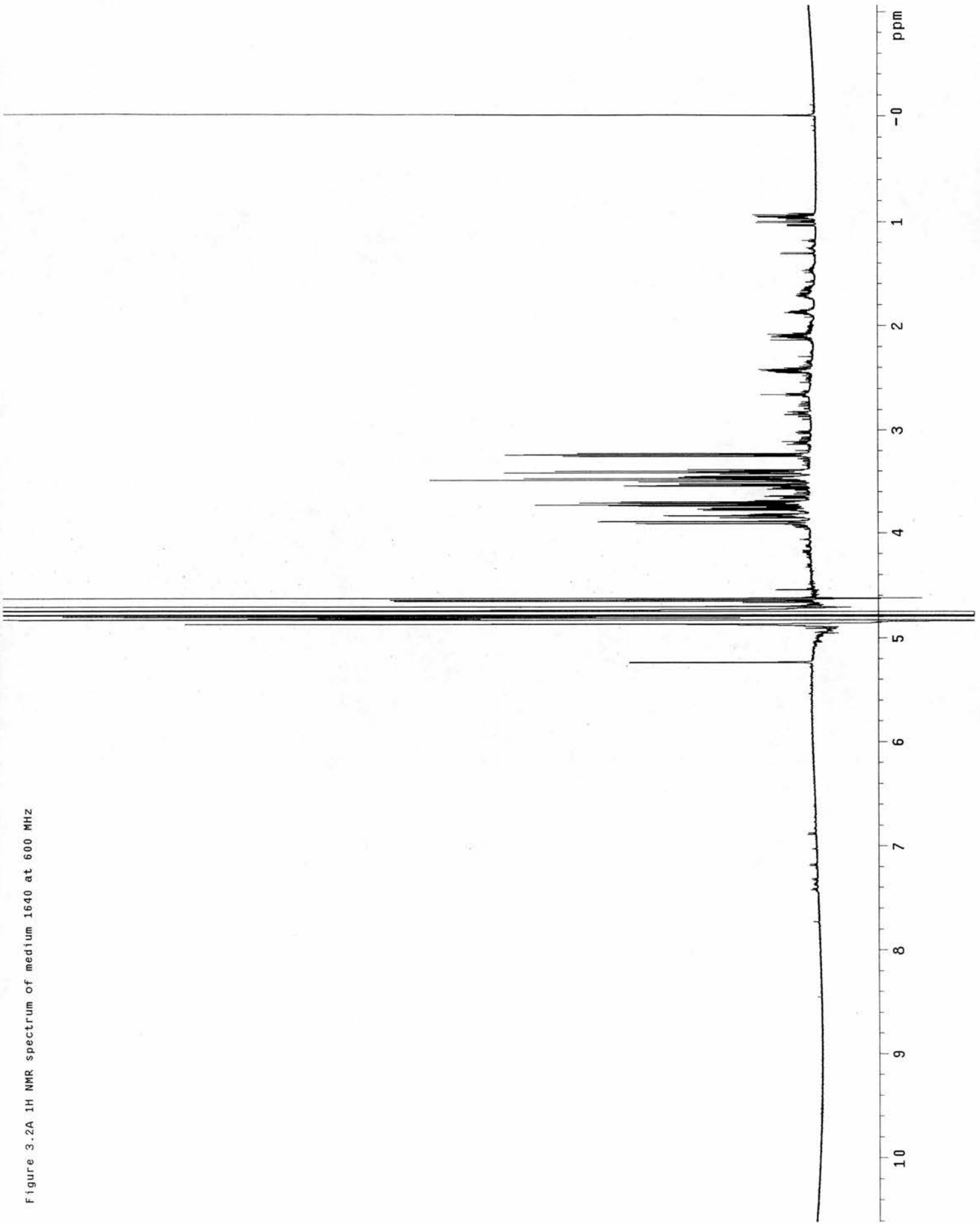


Figure 3.2A 1H NMR spectrum of medium 1640 at 600 MHz

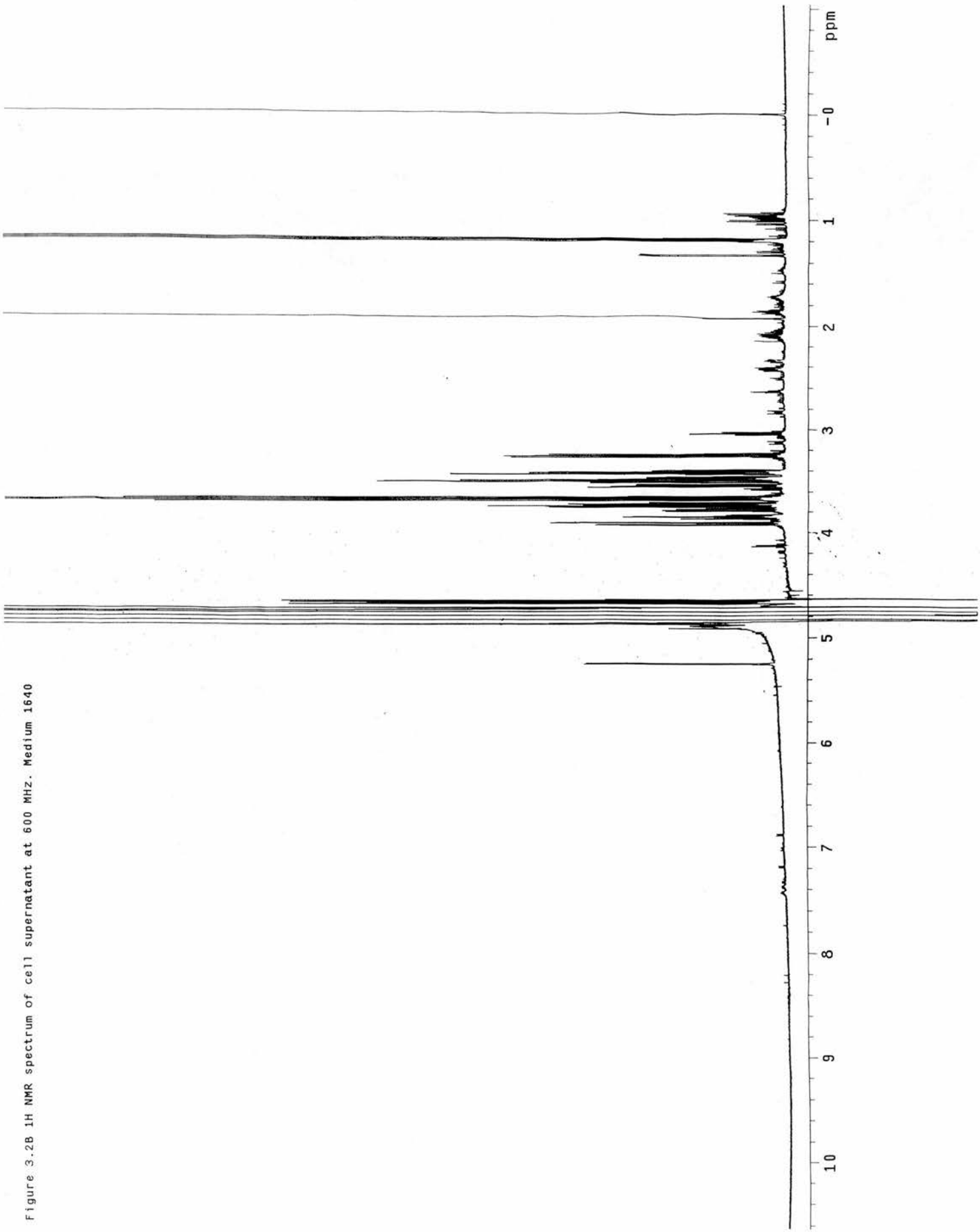


Figure 3.2B 1H NMR spectrum of cell supernatant at 600 MHz. Medium 1640

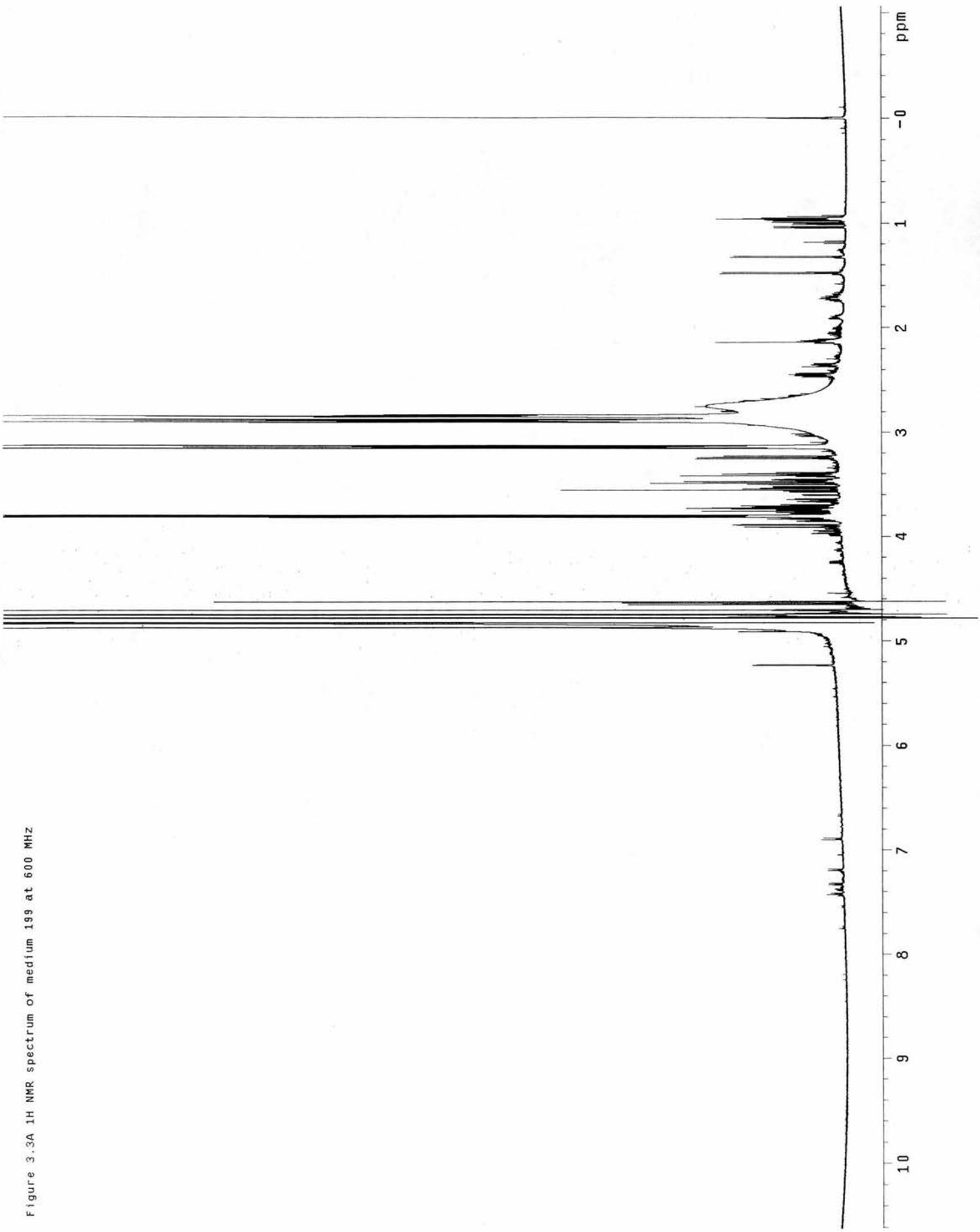


Figure 3.3A 1H NMR spectrum of medium 199 at 600 MHz

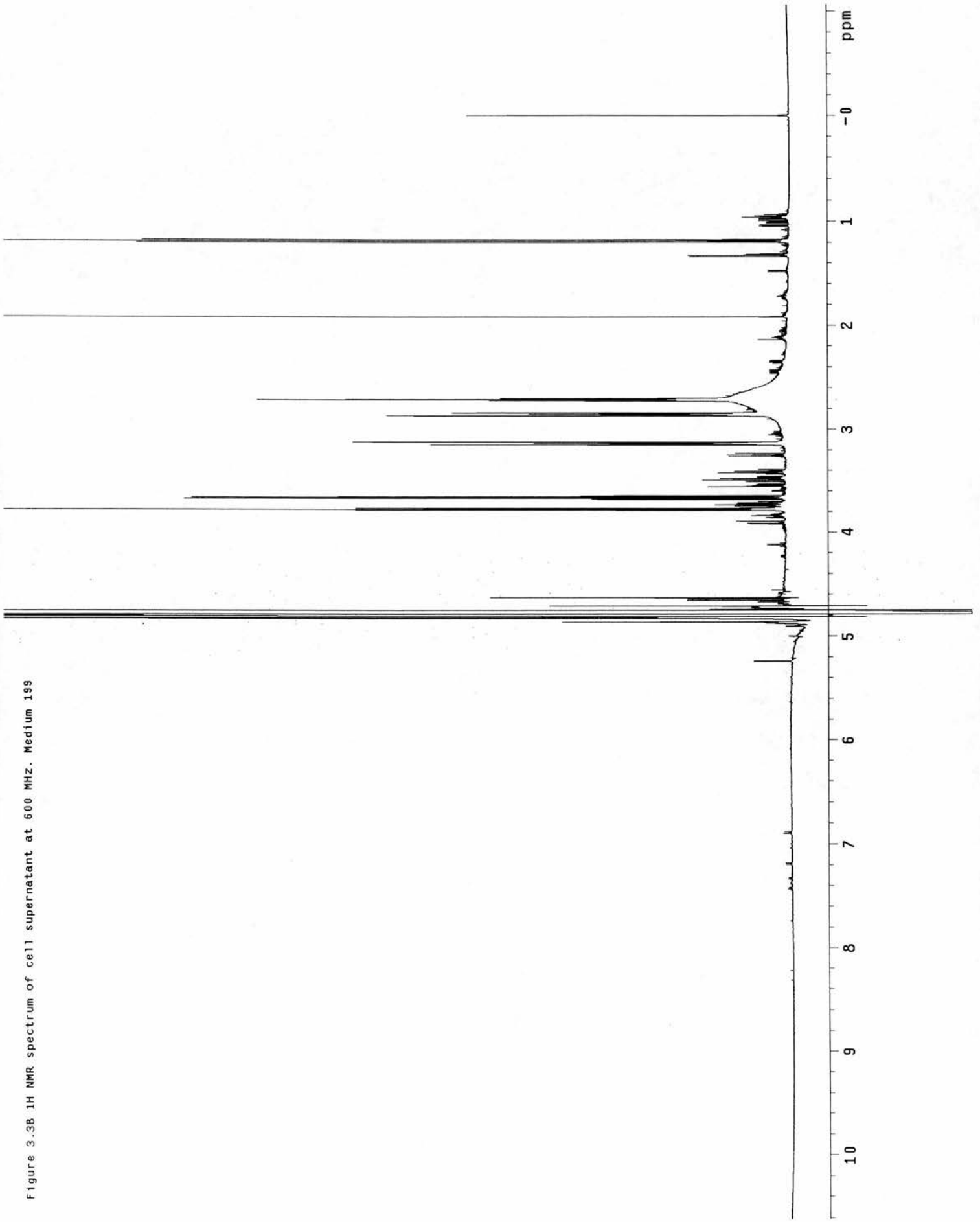


Figure 3.3B 1H NMR spectrum of cell supernatant at 600 MHz. Medium 199

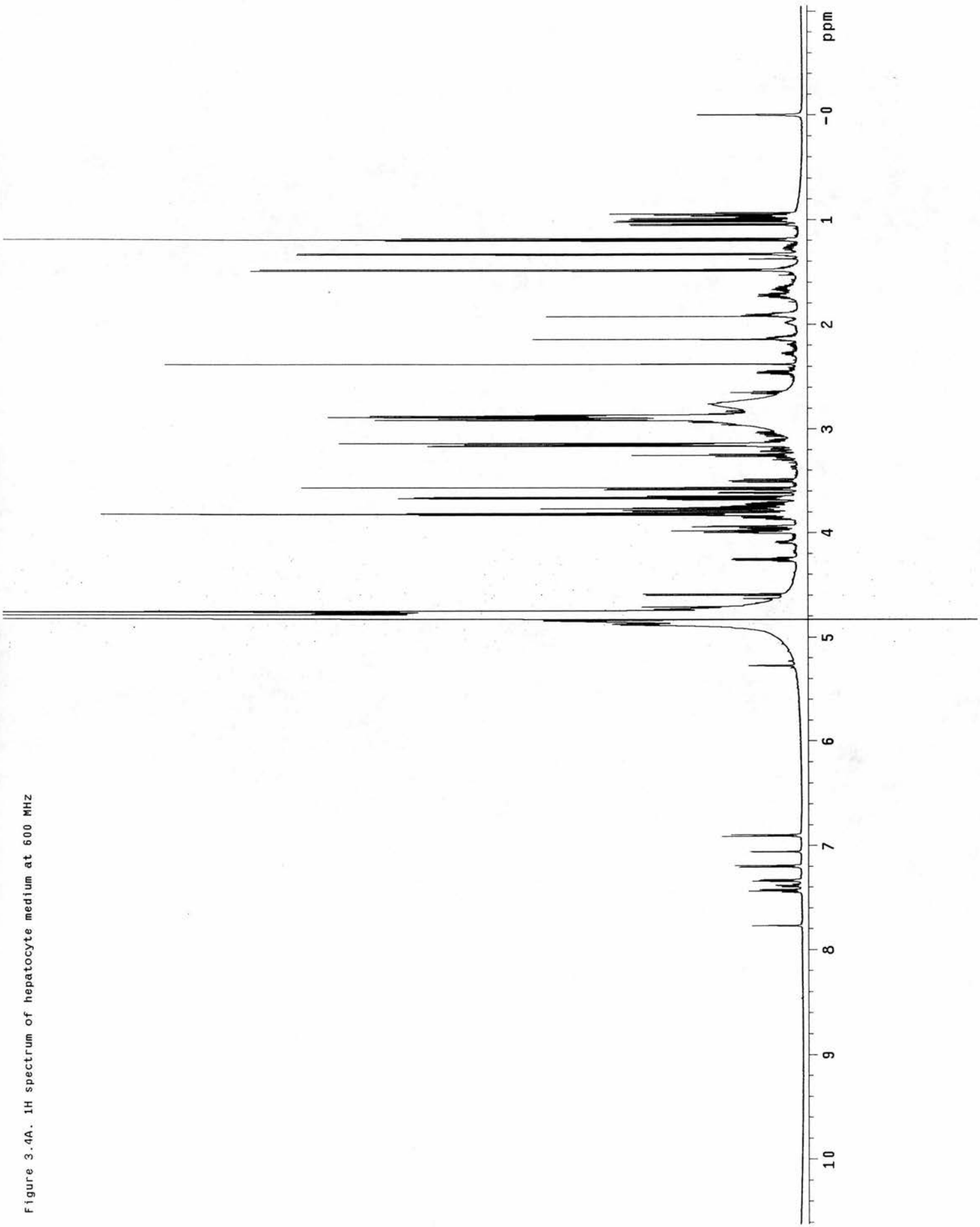


Figure 3.4A. 1H spectrum of hepatocyte medium at 600 MHz

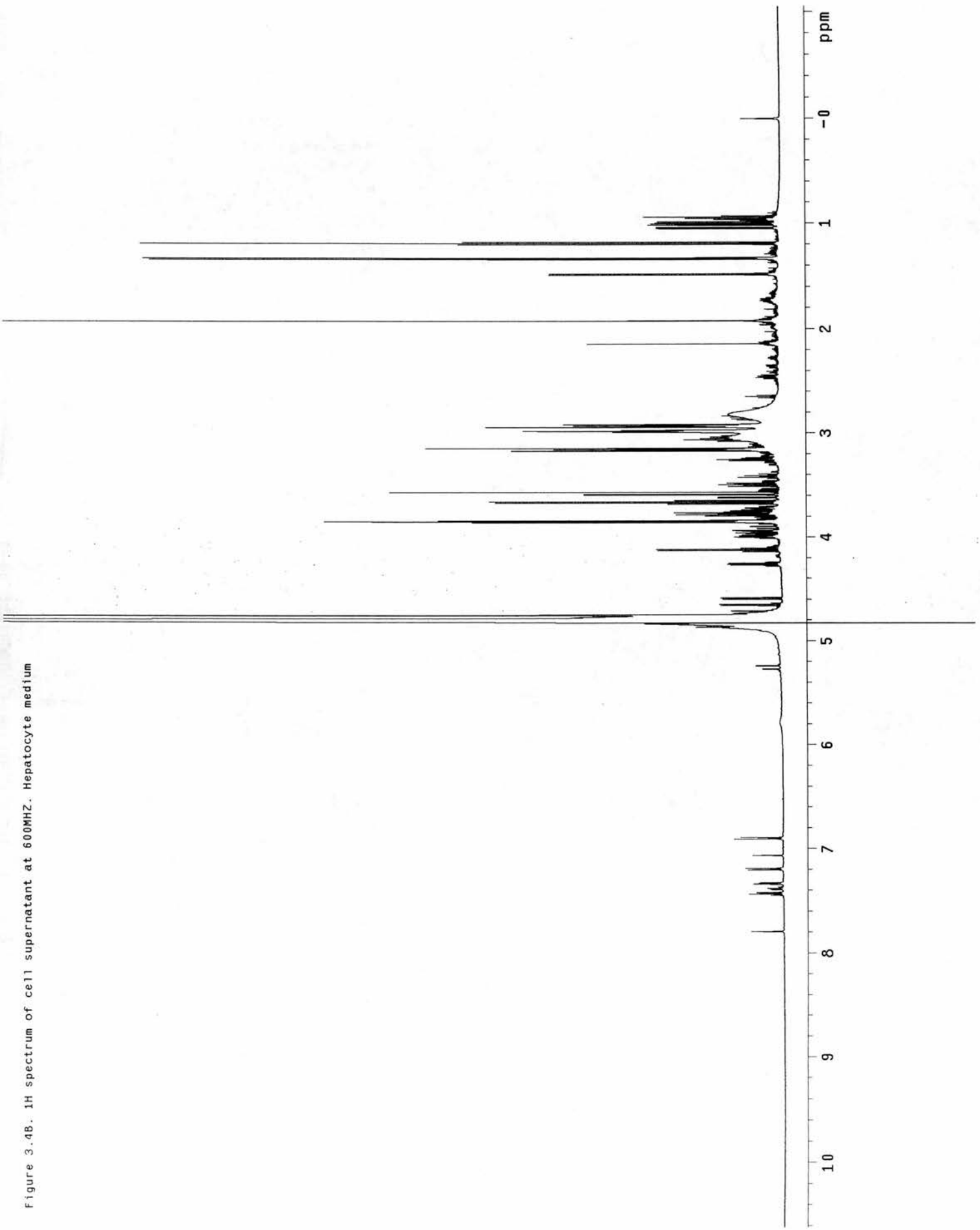


Figure 3.4B. 1H spectrum of cell supernatant at 600MHZ. Hepatocyte medium

3.3.1 GLUCOSE- GALACTOSE

Table 3.2 shows the results obtained for the substances involved in anaerobic glycolysis.

There was consumption of the relevant hexose (glucose in WE, 1640 and 199 media, galactose in HM) in all media and in all days sampled. There was an overall trend in all media, for the consumption of the hexose to be least in day 2, to increase by day 4 and then to more or less plateau by day 6. In media 1640 and 199, there was a statistically significant difference between day 2 and day 4, and day 2 and day 6. (1640 $p<0.015$ between 2 and 4 and $p<0.005$ between 2 and 6. The corresponding figure for medium 199 was $p<0.00005$ for both). Medium 1640 exhibited the smallest overall consumption and HM that contained galactose the greatest hexose consumption.

Between media there were statistically significant differences between medium 1640 and medium 199 in day 4 (bigger consumption in 199, $p<0.0026$) and between medium 199 and WE medium in days 4 and 6 (bigger consumption in 199 $p<0.0006$ and $p<0.0033$ respectively.)

There was evidence of a small glucose production in HM. It is possible to differentiate between glucose and galactose using ^{1}H NMR and this result showed that gluconeogenesis in cells cultured in HM was active.

3.3.2 LACTATE

There was production of lactate under all conditions by hepatocytes cultured in all media, on all days sampled. There was an overall trend for the production of lactate to be least on day 2 and then to increase by day 4. It then dropped by day 6 in all media apart from WE medium, where it rose to its peak concentration by day 6. The drop was significant in HM and there was a statistically significant difference between days 4 and 6 ($p<0.022$). There were statistically significant differences in medium 199 between day 2 and 4 ($p<0.046$) and day 4 and 6 ($p<0.017$). Looking at WE medium there was a statistically significant difference between the smallest production (day 2) and the greatest (day 6) ($p<0.031$).

Between media there were statistically significant differences between medium 1640 and HM on day 6 (bigger production in HM, $p<0.0042$), between medium 1640 and medium 199 on day 6 (bigger production in 199, $p<0.045$), between medium 1640 and WE medium on day 6 (bigger concentration in WE, $p<0.048$), between HM and medium 199 on day 2 (bigger concentration in HM, $p<0.0029$) and between HM and WE medium on days 2 and 4 (bigger concentration in HM $p<0.0012$ and $p<0.012$ respectively). Overall the lowest production was observed with medium 1640 and the highest with HM.

3.3.3 PYRUVATE

The fate of pyruvate was variable depending on the different culture media. In those media that did not contain pyruvate as part of their initial composition (media 1640 and 199) there was a net production of pyruvate at all days sampled. When pyruvate was present initially (media HM and WE) there was a net consumption of pyruvate at all days sampled. As a result there were highly significant statistical differences between medium 1640 and HM on day 2 ($p<0.0000$), on day 4 ($p<0.017$), and day 6 ($p<0.0000$), between medium 1640 and WE medium on day 2 ($p<0.011$), on day 4 ($p<0.0033$) and on day 6 ($p<0.0000$), between medium 199 and HM on day 2 ($p<0.0000$), on day 4 ($p<0.0000$), and on day 6 ($p<0.0000$) and between medium 199 and WE medium on day 2 ($p<0.00008$), on day 4 ($p<0.0000$) and on day 6 ($p<0.0000$). If one compares HM and WE medium there was also statistically significantly more pyruvate consumption in HM by day 4 ($p<0.018$).

Pyruvate production peaked, in both media exhibiting net pyruvate production, on day 2 and then plateaued or slightly decreased. In particular, looking at medium 199, there was a statistically significant difference between days 2 and 4 ($p<0.046$) and days 2 and 6 ($p<0.023$). Pyruvate consumption was least, in both media exhibiting net consumption, in day 2 and peaked by day 4. In particular, looking at WE medium there was a statistically significant difference between day 2 and day 4 ($p<0.008$) and day 4 and day 6 ($p<0.016$).

TABLE 3.2

Percentage changes from basal concentrations of glucose and glucose metabolites in the four different media on days 2, 4 and 6.

Initial concentration for glucose is 7.7 mmols/L in WE and 1640 and 3.88 mmols/L in 199. Galactose concentration in HM is initially 3.5 mmols/L. Initial concentration for pyruvate is 0.2mmols/L in WE and 4.38mmols/L in HM.

	DAY 2	DAY 4	DAY6
		GLUCOSE	
W E	-48 ± 22 %	-60 ± 14 %	-60 ± 17 %
1640	-31 ± 28 %	-62 ± 15 %*	-71 ± 13 %**
199	-48 ± 15 %	-83 ± 13 %**	-82 ± 11 %**
HM	0.19 ± 0.12	0.22 ± 0.11	0.15 ± 0.04
GALACTOSE HM	-62 ± 17 %	-80 ± 8 %	-77 ± 7 %
		LACTATE	
WE	1.07 ± 0.53	1.37 ± 0.59	2.12 ± 0.48
1640	0.40 ± 0.19	0.89 ± 0.69	0.87 ± 0.41
199	1.07 ± 0.51	2.85 ± 2.02*	2.11 ± 0.79*
HM	2.78 ± 1.02	3.13 ± 1.37*	2.02 ± 0.64
		PYRUVATE	
WE	-50 ± 20 %	-85 ± 15 %**	-65 ± 15 %*
1640	0.16 ± 0.11	0.13 ± 0.05	0.09 ± 0.05
199	0.25 ± 0.13	0.09 ± 0.03*	0.08 ± 0.04*
HM	-85 ± 11 %	-94 ± 3 %	-91 ± 7 %

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to the previous day's measurement. Absolute values are in mmols/L

3.3.4 ALANINE

Table 3.3 shows the results obtained from glucogenic amino acids.

Whether the culture medium contained alanine in the first place was a central factor to alanine's fate in our experiments. Medium 1640 did not contain any and although on day 2 there was no production, overall there was a significant production of alanine. There were statistically significant differences between day 2 and day 4 ($p<0.041$) and day 2 and day 6 ($p<0.0053$). WE medium showed a consumption of alanine in days 2 and 4, peaking at day 4 but remarkably an overall production of alanine by day 6. There were statistically significant differences between day 2 and 6 ($p<0.050$) and between day 4 and day 6 ($p<0.034$). Medium 199 showed the biggest consumption in day 2 and the least on day 4. Overall there was significant alanine consumption. HM medium showed a progressively rising alanine consumption that eventually peaks at day 6

Looking for differences between media there were no significant differences apart from between medium 1640 and HM on day 6 ($p<0.022$).

3.3.5 THREONINE

There was consumption of threonine in all media, at all days sampled, apart from day 2 on medium 1640. There was a small consumption on day 2 in all media, which peaked eventually on day 6 in all media, apart from medium 199, where consumption peaked on day 4. There was a statistically significant difference in medium 1640 between day 2 and day 6 ($p<0.015$) and between day 4 and day 6 ($p<0.035$).

The greatest consumption of threonine was exhibited by medium HM. This was followed by medium WE , then medium1640 and then medium 199. There were statistically significant intermedia differences between HM and medium199 on day 2 ($p<0.014$) and on day 6 ($p<0.018$), between HM and medium1640 on day 6 (total consumption in 1640, $p<0.0063$), between WE medium and medium199 on day 6 ($p<0.046$), between medium 1640 and WE medium on day 6 ($p<0.033$) and between 1640 medium and 199 medium on day 6 ($p<0.018$).

3.3.6 ASPARTATE.

Aspartate's fate was also dependent on basal media conditions but only partially. HM medium does not contain aspartate and there was a small steady production of aspartate throughout the experiments. Medium WE showed consumption of aspartate on all days with the smallest on day 6 and the peak on day 4, when there was a total consumption of the existing aspartate. There were statistically significant differences between day 2 and day 4 ($p<0.024$) and between day 4 and day 6 ($p<0.0085$). Medium 199 showed total consumption of aspartate on all days. In contrast medium 1640 showed a big production of aspartate on all days with a peak on day 4.

Between media there were statistically significant differences between medium 1640 and HM on day 6 ($p<0.017$) (higher production by 1640), between HM and WE medium on day 4 ($p<0.0000$), between HM and medium 199 on day 2 ($p<0.034$), on day 4 ($p<0.00007$) and on day 6 ($p<0.0000$), between media 1640 and WE on day 4 ($p<0.033$) and day 6 ($p<0.0068$) and between media 1640 and 199 on day 2 ($p<0.024$) on day 4 ($p<0.038$) and on day 6 ($p<0.00006$).

TABLE 3.3

Percentage changes from basal concentrations of glucogenic amino acids in the four different media on days 2, 4 and 6

Initial concentration of alanine is 0.41mmols/L in WE, 0.39mmols/L in 199 and 3.54mmols/L in HM. Initial concentration of threonine is 0.23mmols/L in WE, 0.12mmols/L in 1640, 0.35mmols/L in 199 and 3.53mmols/L in HM. Initial concentration of aspartate is 0.14mmols/L in WE, and 0.28mmols/L in 199.

	ALANINE		
WE	-76 ± 19 %	-81 ± 17 %*	+14 ± 73 %*
1640	0	0.09 ± 0.05*	0.09 ± 0.04**
199	-79 ± 10 %	-43 ± 18 %	-64 ± 13 %
HM	-26 ± 21 %	-37 ± 39 %	-38 ± 33%
	THREONINE		
WE	-9 ± 17 %	-52 ± 30 %	-52 ± 13 %
1640	+8 ± 50 %*	-8 ± 42 %	-100 %*
199	-6 ± 17 %	-31 ± 40 %	-11 ± 34 %
HM	-49 ± 19 %	-55 ± 22 %	-60 ± 15 %
	ASPARTATE		
WE	-36 ± 45 %	-100 %*	-18 ± 36 %**
1640	+107 ± 153 %	+247 ± 67 %	+140 ± 73%
199	-100 %	-100 %	-100 %
HM	0.23 ± 0.14	0.23 ± 0.11	0.28 ± 0.08

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to the previous day's measurement. Absolute values are in mmols/L

3.3.7 ARGININE

Table 3.4 shows the results for the substances involved mostly in urea and glutamine synthesis and in transamination reactions.

There was consumption of arginine in all media, at all days sampled. Apart from medium HM all exhibited the greatest consumption by day 6. HM showed the biggest consumption on day 2 and then consumption plateaued in slightly smaller levels. The smallest consumption was on day 4 overall apart from WE medium which showed the smallest consumption on day 2. There was one intramedia statistically significant difference in medium 1640 between days 4 and 6 ($p<0.033$). There were no intermedia statistically significant differences but a trend existed for HM and 1640 media to consume more arginine than media WE and 199

3.3.8 HISTIDINE

There was consumption of histidine in all media at all days sampled. The emerging pattern was of a peak of consumption on day 4 in all media, with a decline on day 6. There was a very small consumption on day 2 in medium 1640. There was one intramedia statistically significant difference, in medium 1640 between days 2 and 4 ($p<0.023$).

The greatest consumption was shown by medium 199, followed by media HM, WE and 1640. There were statistically significant intermedia differences between medium 199 and HM on day 2 ($p<0.0000$) and day 4 ($p<0.0061$), between medium 199 and WE medium on day 2 ($p<0.00035$), between media 199 and 1640 on day 2 ($p<0.017$) and between HM and WE medium on day 2 ($p<0.05$).

3.3.9 GLUTAMATE

Overall, there was production of glutamate on all days in all media, except for medium HM on days 4 and 6 where there was a net consumption of glutamate. There were statistically significant differences between day 2 and day 4 ($p<0.041$) and day 2 and day 6 ($p<0.009$) in HM. Looking at medium 199 there was a small overall production of glutamate with a peak on day 2. There were statistically significant differences between day 2 and day 4 ($p<0.048$) and between day 2 and day 6 ($p<0.024$). The production of glutamate was highest in WE medium closely followed by medium 1640.

There were statistically significant differences between media. They were exhibited between media WE and 199 on day 2 ($p<0.0016$) on day 4($p<0.0019$) and on day 6 ($p<0.00001$), between WE medium and HM on day 2 ($p<0.00002$), on day 4 ($p<0.0004$) and on day 6 ($p<0.0000$), between media 1640 and 199 on day 2 ($p<0.001$), on day 4 ($p<0.02$) and on day 6 ($p<0.027$), between medium 1640 and HM on day 2 ($p<0.0001$), on day 4 ($p<0.0088$) and on day 6 ($p<0.011$) and even between HM and medium 199 on day 2 ($p<0.044$) and day 6($p<0.019$).

3.3.10 GLUTAMINE

Glutamine was consumed in all media at all time points sampled. All media contained the same concentration of glutamine as this was added by the study group and that permitted direct comparisons between media. There was not a trend for glutamine consumption seen in all media and there were no statistically significant differences between different days in the same medium.

HM though showed the highest consumption of glutamine followed by WE medium, followed by medium 1640 with medium 199 showing the smallest consumption. There were statistically significant differences between HM and WE medium on day 2 ($p<0.0016$) and day 6 ($p<0.0032$), between HM and medium 1640 on day 2 ($p<0.013$) and day 6 ($p<0.00001$), between HM and medium 199 on day 2 ($p<0.0023$), on day 4 ($p<0.0052$) and on day 6 ($p<0.0000$), between media WE and 1640 on day 2 ($p<0.05$) and day 6 ($p<0.005$), between media WE and 199 on day 2 ($p<0.00008$), on day 4 ($p<0.0000$) and on day 6 ($p<0.0000$) and between media 1640 and 199 on day 2 ($p<0.0001$) on day 4 ($p<0.02$) and on day 6 ($p<0.027$).

TABLE 3.4

Precentage changes from basal concentrations of urea and glutamine synthesis intermediates in the four different media on days 2, 4 and 6

Initial concentration of histidine is 0.07mmols/L in WE, 0.07mmols/L in 1640, 0.1mmols/L in 199 and 1.13mmols/L in HM. Initial concentration of arginine is 0.28mmols/L in WE, 0.21mmols/L in 1640, 0.26mmols/L in 199 and 0.62mmols/L in HM. Initial concentration of glutamate is 0.21mmols/L in WE, 0.29mmols/L in 1640, 0.63mmols/L in 199 and 0.95mmols/L in HM. Initial concentration of glutamine is 1.4mmols/L for all media.

	DAY 2	DAY 4	DAY 6
		HISTIDINE	
WE	-57 ± 29 %	-57 ± 29 %	-43 ± 57 %
1640	-14 ± 43 %	-71 ± 43 %*	-67 ± 14 %
199	-100%	-100%	-60 ± 40 %
HM	-65 ± 10 %	-70 ± 12 %	-67 ± 14 %
		ARGININE	
WE	-57 ± 21 %	-71 ± 25 %	-79 ± 11%
1640	-88 ± 6%	-82 ± 9 %	-100 %*
199	-52 ± 3 %	-46 ± 10%	-43 ± 15 %
HM	-94 ± 15%	-88 ± 5 %	-88 ± 4.5%
		GLUTAMATE	
WE	+200 ± 109 %	+229 ± 138 %	+324 ± 157 %
1640	+351 ± 120 %	+193 ± 152 %	+169 ± 110 %
199	+62 ± 50 %	+8 ± 59 %*	+13 ± 38 %*
HM	+4 ± 41 %	-33 ± 42 %*	-40 ± 26 %**
		GLUTAMINE	
WE	-74 ± 16 %	-54 ± 26 %	-58 ± 19 %
1640	-46 ± 31 %	-62 ± 15 %	-50 ± 25 %
199	-40 ± 25 %	-40 ± 23 %	-31 ± 29 %
HM	-92 ± 3 %	-62 ± 22 %	-95 ± 2 %

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to the previous day's measurement.

3.3.11 LEUCINE

Table 3.5 shows the results for substances involved in ketogenesis.

There was consumption of leucine by all media except medium HM. The consumption was present at all time points sampled in media WE, 1640 and 199. HM exhibited a very small production of leucine overall on days 2 and 6. There was a peak for leucine consumption in all media on day 6, but there were no intramedia statistically significant differences observed.

The biggest consumption was shown by medium 199. Looking at statistically significant differences between the media, differences were exhibited between HM and medium 1640 on day 2 ($p<0.022$), on day 4 ($p<0.023$) and on day 6 ($p<0.0003$), between HM and WE medium on day 2 ($p<0.0000$), on day 4 ($p<0.0044$) and on day 6 ($p<0.0001$), between HM and medium 199 on day 2 ($p<0.0000$), on day 4 ($p<0.00004$) and on day 6 ($p<0.0000$), between media199 and WE on day 2 ($p<0.005$), on day 4 ($p<0.014$) and on day 6 ($p<0.00005$) and between media 199 and 1640 on day 2 ($p<0.017$), on day 4 ($p<0.0025$) and on day 6 ($p<0.001$).

3.3.12 TYROSINE

There was consumption of tyrosine in all media at all days sampled. The smallest consumption was on day 2, apart from medium 199, where the smallest consumption was shown on day 4. There were statistically significant intramedia differences in medium 199 between day 4 and day 6 ($p<0.013$).

Medium 1640 exhibited the greatest overall consumption, followed by WE medium. There were statistically significant differences between media between medium 1640 and HM on day 2 ($p<0.014$), on day 4 ($p<0.016$) and on day 6

($p<0.0033$), between media1640 and 199 on day 2 ($p<0.0061$), between media1640 and WE on day 6 ($p<0.039$), between WE medium and HM on day 4 ($p<0.04$) and between HM and medium199 on day 4 (greater consumption by HM, $p<0.01$) and on day 6 (greater consumption by 199, $p<0.0007$).

3.3.13 PHENYLALANINE

There was consumption of phenylalanine by all media at all days sampled, except by medium 1640 on days 2 and 6, where there was a production of phenylalanine. Consumption of phenylalanine was greatest on day 4 in all media. Overall there were no statistically significant intermedia differences shown.

HM medium exhibited the greatest overall production of phenylalanine. There were statistically significant intermedia differences between HM and WE medium on day 4 ($p<0.042$), between HM and medium 1640 on day 2 ($p<0.011$) and on day 6 ($p<0.042$), between media WE and 1640 on day 2 ($p<0.022$) and between media 199 and 1640 on day 2 ($p<0.011$).

3.3.14 ACETATE

Production of acetate was a phenomenon exhibited in all media and at all time points sampled. There was a pattern emerging with a peak in the production of acetate by all media on day 4. However, there were no statistically significant intramedia differences.

The greatest acetate production was shown by medium 199, followed by WE medium, then by HM and then by medium1640. There were statistically significant intermedia differences between medium 199 and HM on day 4 ($p<0.025$) and on day

6 ($p<0.0086$), between medium 199 and 1640 on day 2 ($p<0.017$), on day 4 ($p<0.013$)and on day 6 ($p<0.0035$), between WE medium and HM on day 2 ($p<0.045$) and on day 4 ($p<0.026$) and between media WE and 1640 on day 2 ($p<0.0058$), on day 4 ($p<0.0073$) and on day 6 ($p<0.036$).

TABLE 3.5

Percentage changes from basal concentrations of ketogenic amino acids and acetate in the four different media on days 2, 4 and 6

Initial concentration of tyrosine is 0.19mmols/L in WE, 0.2mmols/L in 1640, 0.22mmols/L in 199 and 1.16mmols/L in HM. Initial concentration of phenylalanine is 0.14mmols/L in WE, 0.08mmols/L in 1640, 0.21mmols/L in 199 and 1.06mmols/L in HM. Initial concentration of leucine is 0.4mmols/L in WE, 0.27mmols/L in 1640, 0.64mmols/L in 199 and 0.67mmols/L in HM

	DAY 2	DAY 4	DAY 6
		TYROSINE	
WE	-53 ± 21 %	-63 ± 16 %	-58 ± 26 %
1640	-70 ± 20 %	-70 ± 15 %	-80 ± 10 %
199	-45 ± 9 %	-14 ± 14 %	-62 ± 14%*
HM	-34 ± 27 %	-31 ± 26 %	-34 ± 22 %
		PHENYLALANINE	
WE	-16 ± 28 %	-36 ± 14 %	0 ± 21 %
1640	+37 ± 25 %	-13 ± 38 %	+13 ± 25 %
199	-45 ± 24%	-52 ± 3 %	-10 ± 48 %
HM	-37 ± 20 %	-35 ± 17 %	-22 ± 15 %
		LEUCINE	
WE	-53 ± 10 %	-53 ± 20 %	-53 ± 10 %
1640	-12 ± 37 %	-34 ± 34%	-48 ± 22 %
199	-70 ± 11 %	-63 ± 6 %	-72 ± 9 %
HM	0 ± 7%	+4 ± 16%	+3 ± 21 %
		ACETATE	
WE	6.24 ± 2.82	7.01 ± 2.92	6.44 ± 3.10
1640	2.25 ± 1.58	3.51 ± 2.09	2.32 ± 0.96
199	5.42 ± 2.13	8.86 ± 4.28	6.59 ± 2.64
HM	3.61 ± 1.85	4.18 ± 1.95	2.68 ± 1.08

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to the previous day's measurement. Absolute values are in mmols/L

3.3.15 ISOLEUCINE

Table 3.6 shows the results obtained for substances involved mostly in aerobic glycolysis and mostly used as succinate precursors.

There was consumption of isoleucine in all media, at all days sampled. There was a trend for all media to show the smallest consumption by day 2 but there were no statistically significant intramedia results. Consumption was then increased and reached its peak at day 4 for media WE and 1640 and at day 6 for media 199 and HM.

All media consumed fairly similar amounts of isoleucine at all days, the exception being medium 199, which exhibited a significantly higher consumption than the other media on day 6. This reached statistical significance when compared with medium HM ($p<0.011$) and medium WE ($p<0.0007$).

3.3.16 VALINE

There was a small consumption of valine by all media, at all days sampled. The consumption increased as time goes by and it peaked on day 6 for all media, except for WE medium, where the consumption was constant on all days. There were no statistically significant intramedia differences observed.

Overall the greatest consumption was shown by HM medium but there were no statistically significant differences except when comparing HM with WE medium (significantly smaller by WE) on day 4 ($p<0.016$) and on day 6 ($p<0.00042$). Differences between medium 199 and WE medium reached also statistical significance on day 6 (less consumption by WE, $p<0.0007$).

3.3.17 METHIONINE

There was consumption of methionine in all media at all days sampled. The consumption peaked on day 6 in all media except in medium WE where methionine consumption peaked on day 4 to a total consumption of the methionine contained in the medium. There were no statistically significant intramedia differences however. Looking at differences between media there was only one present between WE medium and HM on day 2 ($p<0.033$) and on day 4 ($p<0.0000$). Overall there was an increase in methionine consumption as time went by culminating at a peak on day 6. There was a trend for medium WE to exhibit the greatest overall consumption and for medium HM the smallest.

3.3.18 ETHANOL

Table 3.7 shows the results of ethanol production by the different media. There was ethanol production in all media at all days sampled. There was a uniform pattern in all media with a peak production on day 2, a small decrease on day 4 and a further decrease on day 6, except for medium 199 where production was maximum on day 4 but minimum on day 6. Looking at statistically significant intramedia differences these were present in all media. In medium 1640 between day 2 and day 4 ($p<0.0044$) and between day 4 and day 6 ($p<0.039$), in medium 199 between day 2 and day 6 ($p<0.01$), in medium HM between day 2 and day 6 ($p<0.034$) and in medium WE between day 2 and day 6 ($p<0.027$). There were no significant intramedia differences as all media exhibited the same pattern of ethanol production.

TABLE 3.6

Percentage chenges from basal concentrations of succinate precursors in the four different media on days 2, 4 and 6.

Initial concentration of isoleucine is 0.27mmols/L in WE, 0.27mmols/L in 1640, 0.21mmols/L in 199 and 1.33mmols/L in HM. Initial concentration of valine is 0.3mmols/L in WE, 0.12mmols/L in 1640, 0.3mmols/L in 199 and 1.71mmols/L in HM. Initial concentration of methionine is 0.07mmols/L in WE, 0.07mmols/L in 1640, 0.14mmols/L in 199 and 0.7mmols/L in HM.

	DAY 2	DAY 4	DAY 6
		ISOLEUCINE	
WE	-27 ± 26 %	-33 ± 30 %	-22 ± 22 %
1640	-7 ± 30 %	-41 ± 26 %	-37 ± 30 %
199	-34 ± 19 %	-38 ± 19 %	-57 ± 10 %
HM	-22 ± 20 %	-35 ± 23 %	-38 ± 19 %
		VALINE	
WE	-27 ± 20 %	-27 ± 26 %	-20 ± 20 %
1640	-8 ± 25 %	-8 ± 25 %	-34 ±17 %
199	-20 ± 17 %	-36 ± 20 %	-50 ± 7 %
HM	-47 ± 16%	-51 ± 18 %	-54 ± 15 %
		METHIONINE	
WE	-71 ± 14 %	-100 %	-43 ± 14 %
1640	-58 ± 42 %	-29 ± 42 %	-58 ± 29 %
199	-36 ± 4 %	+43 ± 5 %	-71 ± 21 %
HM	-19 ± 16 %	-6 ± 26 %	-20 ± 33 %

Results shown are mean ± SEM.

TABLE 3.7

Concentrations of ethanol in the four different media on days 2, 4 and 6 (mmols/L)

ETHANOL

	DAY 2	DAY 4	DAY 6
WE	11.86 ± 7.02*	5.11 ± 2.09	3.19 ± 2.61
1640	8.89 ± 4.22	5.21 ± 1.86**	2.87 ± 1.60*
199	10.57 ± 4.37*	13.66 ± 11.88*	2.19 ± 0.78
HM	5.22 ± 2.15	5.09 ± 3.05	2.56 ± 1.17

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to the previous day's measurement.

3.4 DISCUSSION

This study deals with the following amino acids that can be assigned on NMR spectroscopy using 1D and 2D techniques. Alanine, threonine, aspartate, phenylalanine, tyrosine, glutamate, glutamine, valine, leucine, isoleucine methionine, histidine and arginine. Some signals from amino acids were obscured by the water signal which is remarkably high even when suppressed. Signals from other amino acids overlap with powerful signals from molecules present in high concentrations like glucose. Unfortunately, all those amino acids could not be identified.

It is well known that amino acids can be produced by the cell from different intermediate substances as part of the anabolic process of the cell. But cells are also constantly breaking down proteins releasing amino acids. The final concentration of those amino acids will depend on both those processes. It appears that as cell were under the same experimental conditions, the contribution of protein breakdown to the final amino acid concentration was equivalent in all culture media.

This study produces some evidence that the culture medium although it does not influence the survival of the hepatocytes cultured can influence their metabolic profile. Researchers who want to enhance or suppress a particular pathway can do so by changing the concentration of amino acids in solution i.e. the culture medium itself.

3.4.1 William's E Medium

William's E Medium showed a predominantly anaerobic pattern of metabolism. This was manifested by a high glucose consumption and also a high production of lactate. There was breakdown of pyruvate and a small consumption of alanine , aspartate and threonine. The overall consumption of gluconeogenic amino acids was an indication that gluconeogenesis was active. We have also shown that WE medium exhibited a moderate consumption of valine, methionine and isoleucine. This was an indication that the Kreb's cycle for aerobic ATP production was not very well fed. The glycolysis in cells cultured in WE was far from the ideal scenario of aerobic metabolism with small lactate production and small pyruvate consumption. The ketogenic amino acids leucine, isoleucine, phenylalanine and tyrosine exhibited a moderate to high consumption. The fact that they were used in ketogenesis was proven by the big production of acetate. High glutamate production indicated that transamination was very active. High histidine consumption pointed to a production of glutamate from histidine, while high arginine consumption was indicative of a functioning urea cycle. A lot of other substrates able to feed the urea cycle were present in the cells so the small diversion of glutamate to aspartate came as no surprise. High consumption of glutamine implied that cells were actively using it for DNA synthesis and cellular division.

3.4.2 Medium 1640

Medium 1640 on the contrary, showed a pattern of aerobic ATP production with small lactate production ,small increase in pyruvate and alanine concentration, small threonine consumption and a high glucose consumption. The pattern overall was of inactive gluconeogenesis. Succinate precursors valine, isoleucine and methionine exhibited a moderate consumption but the overall pattern of metabolism supported the view of a well fed Kreb's cycle. There was a small acetyl -CoA production which was to be expected as there was a small consumption of leucine and isoleucine and no consumption of phenylalanine. Although we mentioned that ketogenesis from amino acid break down is only an alternative pathway, it is indicative of a less active process. There was a very high tyrosine consumption that could account for some of the acetyl-CoA generation. Aspartate concentration increased sharply indicating that although aspartate was produced from glutamate it was not converted to either alanine or arginine. Aspartate was then incorporated either into the urea cycle or into the Kreb's cycle. The urea cycle was active with a high histidine consumption and a very high arginine consumption and that would fit with the high aspartate consumption. Glutamate concentration increased sharply showing active transamination with a matching glutamine consumption. Glutamine is essential in cell division and cells tend to use glutamine when they are actively replicating.

3.4.3 Medium 199

Medium 199 exhibited a high glucose consumption accompanied by a high alanine consumption and a moderate lactate production. As pyruvate was not present in basal condition there was a small overall pyruvate production. There was also a small production of threonine. Diversion of aspartate to alanine with production of pyruvate was indicative of active gluconeogenesis. An active Kreb's cycle was manifested by a high valine and methionine consumption accompanied by some isoleucine consumption. All those amino acids can feed the Kreb's cycle through production of succinate. There was a very high consumption of aspartate with a small overall production of glutamate indicating that the urea cycle was active. This was also manifested by the high rate of catabolism of histidine and arginine. The small production of glutamate was an indication that transamination was less active than in other media. There was also a small glutamine synthesis indicating that DNA synthesis has slowed down under those conditions. There was a high acetyl-CoA production although there is moderate consumption of all four ketogenic amino acids. As we mentioned earlier the bulk of ketone bodies is produced from fatty acid oxidation and it seems that this pathway was active under those conditions.

3.4.4 Hepatocyte Medium

There are essential differences in the constitution of Hepatocyte Medium (HM) and the other three media examined. HM is a very rich medium and the concentrations of all the amino acids in basal conditions are much higher in HM than in other media. It also contains galactose as the hexose to be used as fuel for the cell. HM exhibited a very high galactose consumption with a high lactate production and a

small alanine consumption. This indicated an anaerobic pattern of metabolism which was further underlined by a small aspartate production and a small phenylalanine and tyrosine consumption. The presence of glucose in the supernatants was indicative of gluconeogenesis. In contrast to other media there was consumption of glutamate accompanied by a high glutamine consumption indicating a huge DNA synthesis but not enough transamination, although both histidine and arginine are consumed. All glutamate produced was transformed to glutamine. There was very small methionine and isoleucine consumption and although there was moderate valine consumption we can conclude that a small amount of succinate was produced and that Kreb's cycle was not very active and well fed. There was moderate production of acetate with all ketogenic amino acids exhibiting small consumption. Leucine in particular was not being consumed at all. It is supposed that fatty acid oxidation compensates for the small amino acid consumption to produce a moderate amount of acetate.

Overall we have evidence to support the hypothesis that the culture medium plays an important role on how the cultured cells will behave during the experiments. In WE and HM there was an anaerobic metabolic pattern exhibited which was less prominent in 199 and turned into aerobic predominantly in medium 1640. The fact that key substances like pyruvate and alanine were missing in medium 1640 might be the key to this slow aerobic process exhibited. Also, WE and 1640 exhibited well functioning transamination reactions with a lot of glutamate production which was then diverted either to urea synthesis or glutamine. In contrast 199 and HM exhibited very little transamination. The urea cycle was not performing very well with HM although in 199 the fact that different amino acids could feed it compensated for the small transamination. In 199 there was evidence of a small glutamine consumption

which shows that its utilisation in DNA synthesis and the division process was not very successful. The other media showed that DNA synthesis was active to a great extent. Acetyl -CoA synthesis was present in all media with WE and 199 showing a lot of acetate production followed by AM and finally 1640 which showed relatively small acetate production. As both fatty acids and ketogenic amino acids can be the source for acetate we can only monitor the ketogenic amino acids and we think this is why , although it seems that ketogenic amino acid consumption by all media was roughly similar, we observed differences in the final product concentration.

This study produces evidence that it is possible to direct cells towards an aerobic or anaerobic metabolic pattern. This can be done not only by supplying enough oxygen but also by changing the concentrations of the amino acids present in the cell culture medium. Further studies are needed to investigate the mechanisms that control the flow of substances in hepatocytes in order to have a better understanding of the above described results.

CHAPTER 4

STUDIES ON CRYOPRESERVED HEPATOCYTES

4.1 INTRODUCTION

Fulminant hepatic failure (FHF) is a life - threatening condition that may require a liver transplant (Plevris et al, 1998). Lack of availability of donor livers means that these patients will die while awaiting for a liver transplant.

Bioartificial liver support systems (BALSS) have long been regarded as a desirable therapeutic modality to bridge patients to transplantation or even to allow for complete regeneration of the patients' livers (Gerlach et al, 1996; Demetriou et al, 1998a). Currently, a few centres around the world have been developing and testing BAL systems either on animal models of liver failure or patients, with encouraging results (Sussman et al,1992; Clement et al, 1996; Janke et al, 1997; Watanabe et al,1997; Samuel et al, 1997; Ijima et al, 1998). Most available data at present suggest that BAL systems can, despite their limitations, successfully bridge patients to transplantation (Sussman et al, 1992; Watanabe et al, 1997; Samuel et al, 1997).

Hepatocyte transplantation is another exciting new modality to treat FHF. Hepatocytes are infused into the spleen or the portal system of the patient in order to provide liver support temporarily, allowing for regeneration of the injured liver (Nakamura et al, 1997; Arkadopoulos et al,1998; Fox et al, 1998).

Due to the scarcity of human hepatocytes, primary porcine hepatocytes are currently regarded as the cell of choice for liver support. They are metabolically active with similar biochemical profile to human hepatocytes and can be obtained in

the large numbers that are required for BAL treatment and hepatocyte transplantation (Jauregui et al, 1997; Demetriou et al, 1998b; Clement et al, 1998). Provision of porcine hepatocytes, on demand, to treat FHF patients is logistically difficult; a feasible way therefore to have primary porcine hepatocytes available on demand would be to cryopreserve them and to thaw them as soon as the need arises (Naik et al, 1997; Sheil et al, 1998). However, although this approach has been used by several available BAL support systems, data regarding viability and plating efficiency show that cryopreservation inversely affects those parameters (Chesne et al, 1993; Naik et al.1997) Detailed metabolic studies of cryopreserved hepatocytes are lacking.

So far studies evaluating the metabolic functions of cryopreserved hepatocytes have been limited to monitor enzyme activities, drug and xenobiotic metabolism and albumin production, because the techniques to measure the production of substrates from the different biochemical cycles that are ongoing into the hepatocytes are not widely available(Sun et al, 1990; Kasai et al, 1993; Diener et al, 1993; Koebe et al, 1996, Naik et al, 1997,).

The aim of this study was to systematically investigate the effect of different protocols of cryopreservation on primary porcine hepatocyte viability and metabolism using ^{1}H Nuclear Magnetic Resonance Spectroscopy.

4.2 MATERIALS AND METHODS

Hepatocyte isolation was performed as previously described in chapter 3

4.2.1 Cell culture

Both fresh and cryopreserved hepatocytes were seeded at a density of 8.10^6 cells / plate (90 mm standard cell culture dishes) (Corning Inc.). Collagen coated plates were used to evaluate the effect of collagen coating on viability and plating efficiency of cryopreserved hepatocytes. 150µl of diluted type I (rat tail) collagen was used and plates were dried overnight under aseptic conditions before hepatocyte plating (Sun et al, 1990). The culture media used were supplemented Williams E medium or Medium 199. Media were used both as culture and as cryopreservation media to evaluate the effect of different media on cryopreservation. These media were hormonally defined and supplemented as in Chapter 3. The culture medium was renewed every day during the culture period. Cultures were maintained under an atmosphere of 95% air and 5% CO_2. They were routinely examined under phase contrast microscopy before every culture medium renewal. If media were used as cryopreservation media then 10 % De_2MO was used as cytoprotectant (Diener et al, 1993). 10% Foetal bovine serum was also evaluated as an alternative to supplements for the cryopreservation experiments (Lawrence et al, 1991).

4.2.2 Microcarrier preparation.

Cytodex microbeads were evaluated as an alternative attachment substrate. Type 3 microbeads were used. They were prepared according to manufacturer's instructions (Microcarrier cell culture; ptrinciple and methods, Pharmacia LKB Biotechnology,1995). Briefly, they were soaked in Phosphate Buffer Solution using aseptic techniques and were then sterilised in a wet autoclave. After isolation and purification cells (10^6 cells/ml) were transferred to microbeads suspensions (10^4

microcarriers/ml). They were left overnight in the incubator at 37^{0}C to optimise attachment conditions. They were then cryopreserved using protocol VIII.

4.2.3 Cryopreservation protocols

Eight different cryopreservation protocols were used. They were divided into slow freezing protocols, quick freezing protocols, stepwise freezing protocols and protocols for the cryopreservation of plated hepatocytes.

Quick freezing protocols were as follows:

Protocol I: -5^{0}C/min from +4^{0}C to -100^{0}C (Lawrence et al, 1991)

Protocol II: -30^{0}C/min from +4^{0}C to -100^{0}C(Lawrence et al, 1991)

Slow freezing protocols were as follows:

Protocol III: -2^{0}C/min from +4^{0}C to -100^{0}C

Protocol IV: -0.5^{0}C/min from +4^{0}C to -100^{0}C

Stepwise freezing protocols were as follows:

Protocol V: At 4^{0}C for 20 minutes; -1^{0}C/min from 4^{0}C to -4^{0}C; -25^{0}C/min from -4^{0}C to -40^{0}C; +10^{0}C/min from -40^{0}C to -12^{0}C; -1^{0}C/min from -12^{0}C to -40^{0}C; -10^{0}C/min from -40^{0}C to -100^{0}C (Diener et al, 1993).

Protocol VI: -2.5^{0}C/min from 4^{0}C to 0^{0}C; 8 minutes at 0^{0}C; -2^{0}C/min from 0^{0}C to -8^{0}C; in 10 seconds down from -8^{0}C to -28^{0}C; -2.5^{0}C/min from -28^{0}C to -33^{0}C; +1.5^{0}C/min from -33^{0}C to -27^{0}C; -2^{0}C/min from -27^{0}C to -60^{0}C; -10^{0}C/min from -40^{0}C to -100^{0}C (Sun et al, 1990).

Protocol VII: At 4^{0}C for 15 minutes; -1^{0}C/min from +4^{0}C to -5^{0}C; -3^{0}C/min from-5^{0}C to -12^{0}C; -5^{0}C/min from -12^{0}C to -14^{0}C; -7.5^{0}C/min from -14^{0}C to -20^{0}C; -6.5^{0}C/min

from -20^{0}C to -25^{0}C; at -25^{0}C for 4 minutes; +3^{0}C/min from -25^{0}C to -20^{0}C; at -20^{0}C for 2 minutes; -1^{0}C/min from -20^{0}C to -50^{0}C; -10^{0}C/min from -50^{0}C to -100^{0}C.

Cryopreservation protocols used for plated hepatocytes were as follows:

Protocol VIII: -0.3^{0}C/min from +4^{0}C to -100^{0}C.

Protocol IX: At room temperature for 20 min. At 4^{0}C for 30 min. At -30^{0}C for 90 min. Then stored at -70^{0}C.

4.3 Cryopreservation

Cells were cryopreserved either as ordinary suspensions, suspensions attached to microbeads or attached on plates.

4.3.1 Cell suspensions

After evaluation of the viability of the freshly isolated hepatocytes using the trypan blue exclusion method, cells were again centrifuged (25g for 5 min) twice and the pellet resuspended in culture medium. DMSO was introduced slowly over 4 minutes to a final concentration of 10% v/v. The final concentration was 2.10^6 cells/ml of cryopreservation solution. Suspensions were kept in 2 ml Nunc cryovials containing approximately 1.5 ml of suspension. They were then cryopreserved using cryopreservation protocols I- VIII.

Foetal bovine serum (FBS) as an adjunct to the cryopreservation media was evaluated using protocols I, VI and VIII.

4.3.2 Plated hepatocytes

They were cultured on standard and collagen coated culture dishes until they reached confluence at day 2 post isolation. William's E medium and medium 199 were evaluated as incubation media. The supernatant was then aspirated and replaced with a thin film of culture medium containing 10% v/v De_2MO. They were then cryopreserved using cryopreservation protocols VIII and IX.

All cells were stored in liquid nitrogen (LN_2) at -196^{0}C. After 7 days of cryopreservation in LN_2 cells were thawed using the following protocol. They were gently shaken in a water bath at 37^{0}C. Immediately after liquefaction of the culture medium, the supernatant was aspirated and cells were centrifuged at 25g for 5 minutes and washed three times with HBSS buffer. Cells suspensions were then plated on standard Petri dishes or on collagen coated Petri dishes and incubated. Cells already plated were placed in the incubator after replenishment of the culture medium. Viability was assessed immediately post thawing using the trypan blue exclusion test. Morphology and plating efficiency were assessed at 24 h post thawing using the % LDH test (Marsh et al, 1991.) and morphology of the cells under light microscopy.

4.3.3 Microcarriers

Cells attached to microcarriers were thawed using the same method. They were then detached from the microcarriers using trypsin as per the manufacturer's instructions and plated as per normal cryopreserved cells.

Proton NMR spectroscopy experiments were performed as previously described in Chapters 2 and 3. Assignments were made for the following substances.

Glucose, lactate, pyruvate, alanine, glutamate, glutamine, arginine, aspartate, leucine, isoleucine, valine, tyrosine, histidine, phenylalanine, acetate and ethanol

4.3.4 Statistical analysis

Comparisons between freshly isolated and cryopreserved hepatocyte from the same isolation were made. Student's t-test was used to compare means of different groups. A p value of < 0.05 was taken as significant (two-tail test of significance). To compare between more than two groups we used the Tukey test based on the one factor independent measures ANOVA. Results are expressed in mmols/L and are corrected by the number of viable cells per dish

4.4 RESULTS

4.4.1 Cell morphology

Figure 4.1 shows the contrast phase light microscopic appearance of freshly isolated cells incubated for 48h and Figure 4.2 the appearance of cryopreserved cells frozen for 1 week and then plated, after a 24 h incubation. The colonies of cryopreserved hepatocytes did not show spreading of the cells on the plates and the cells themselves showed poor morphology with extensive vacuole formation.

4.4.2 Cells cryopreserved on microcarriers

Although Cytodex 3 microcarriers which are the optimal type of microcarriers for hepatocytes were used and the protocols rigorously observed it was evident that the attachment of cells on the microcarrier beads was minimal. Light microscopy of cells incubated with microcarriers revealed a few cells trapped between microbeads but most cells unattached to the microcarriers. No microbead was observed covered with hepatocytes which is a sign of optimal attachment. The viability of hepatocytes cryopreserved on microcarriers was between 5% and 8% using both cryopreservation protocols. Consequently, experiments on microcarriers were abandoned early in the study.

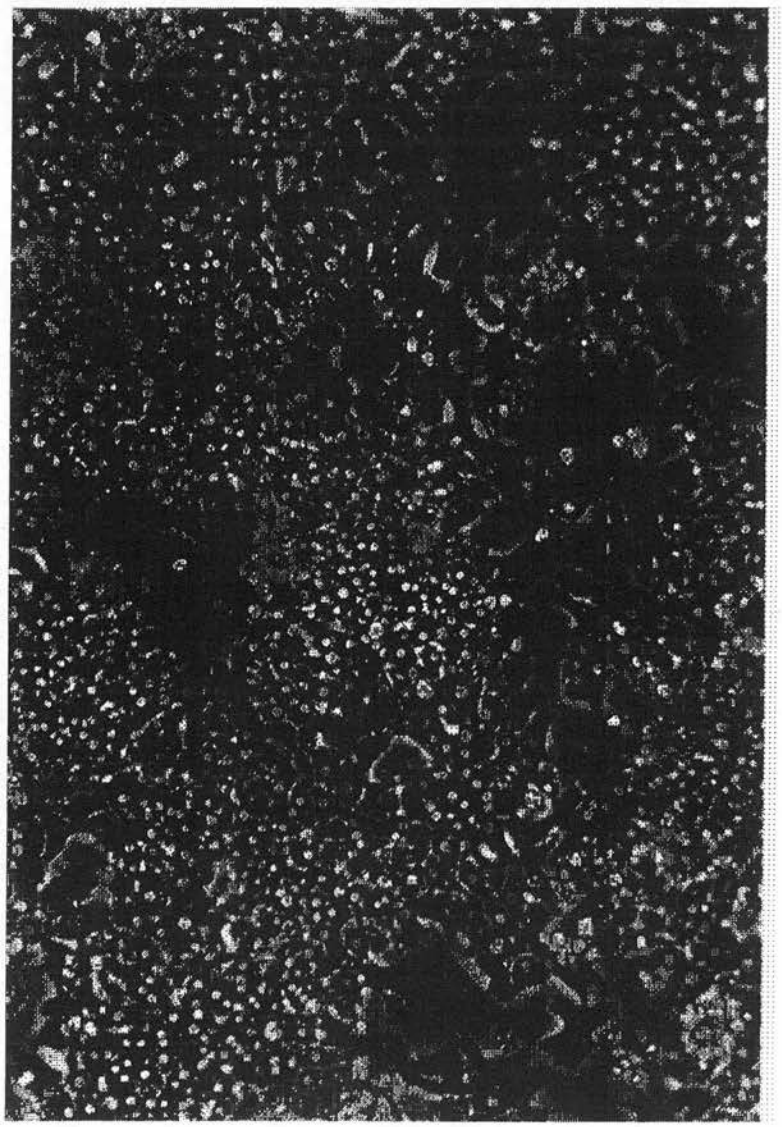


Figure 4.1 Inverted phase light microscopy appearances of primary porcine hepatocytes at 24 h in culture in normal conditions

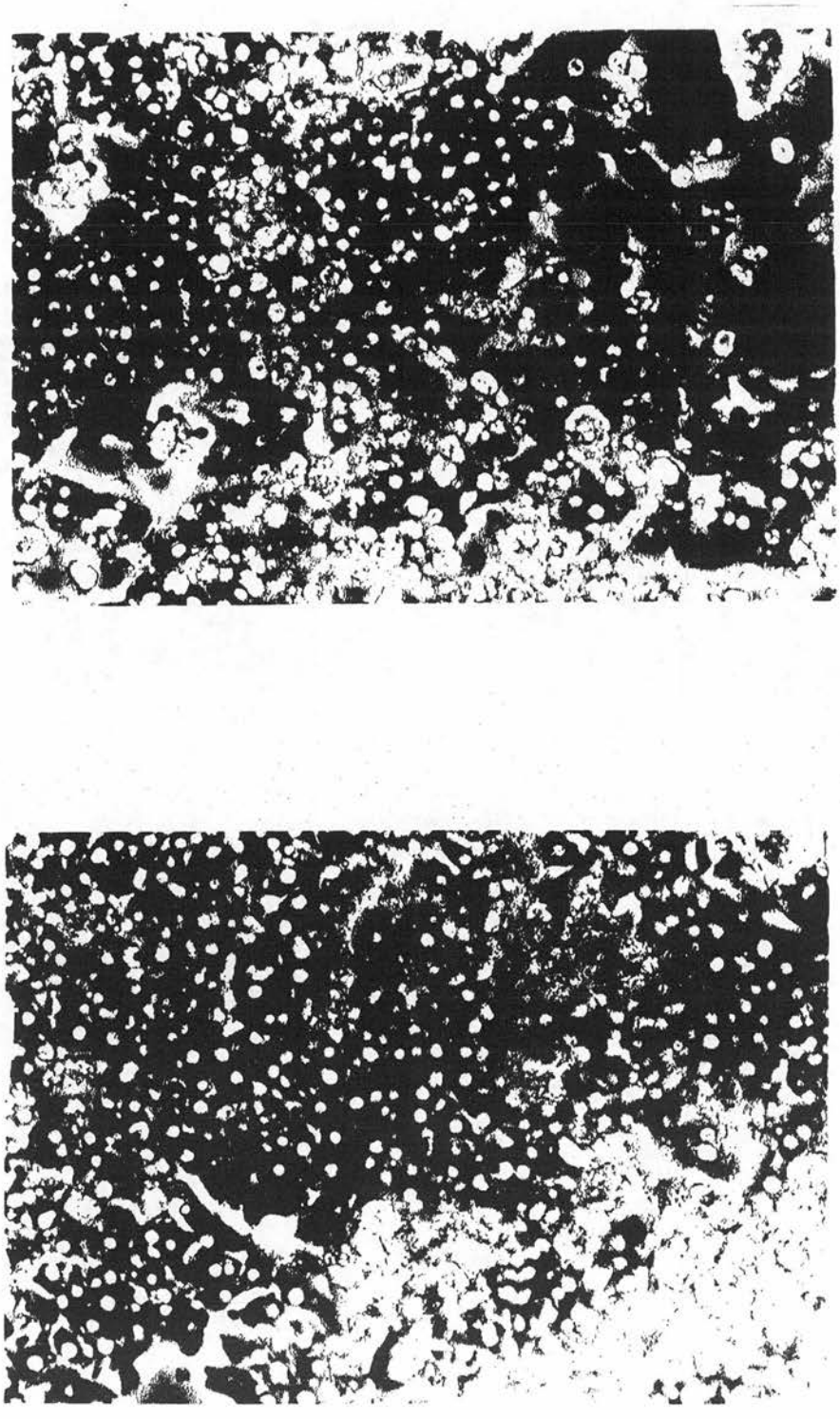


Figure 4.2 Inverted phase light microscopy appearances of primary porcine hepatocytes after 24 h in culture after 7 days in cryopreservation.

4.4.3 NMR Results.

Figure 4.3 shows a single pulse 600 MHz ^{1}H NMR spectrum from cell supenatants from cultured cryopreserved hepatocytes on plates at 24 h post plating. Peaks were assigned to low molecular weight substances, as described in Chapter 1.

4.4.4 Effect of foetal bovine serum.

Experiments were conducted on four different isolations. Viability was significantly better when no FBS was used ($p<0.032$) but no difference was found with regards to plating efficiency (Table 4.1). Glutamate production and glutamine breakdown were significantly more active in cells cryopreserved without FBS ($p<0.05$ and $p<0.04$ respectively) (Figure 4.4). No significant differences were observed in glutamine, succinate and acetate precursors uptake or in glucose metabolism indices. Acetate production by cells preserved without FBS was higher when compared with cells preserved under the same cryopreservation protocol but with FBS. The differences in protocol VIII reached statistical significance ($p<0.03$) (Figure 4.4). All further experiments were conducted using non supplemented media.

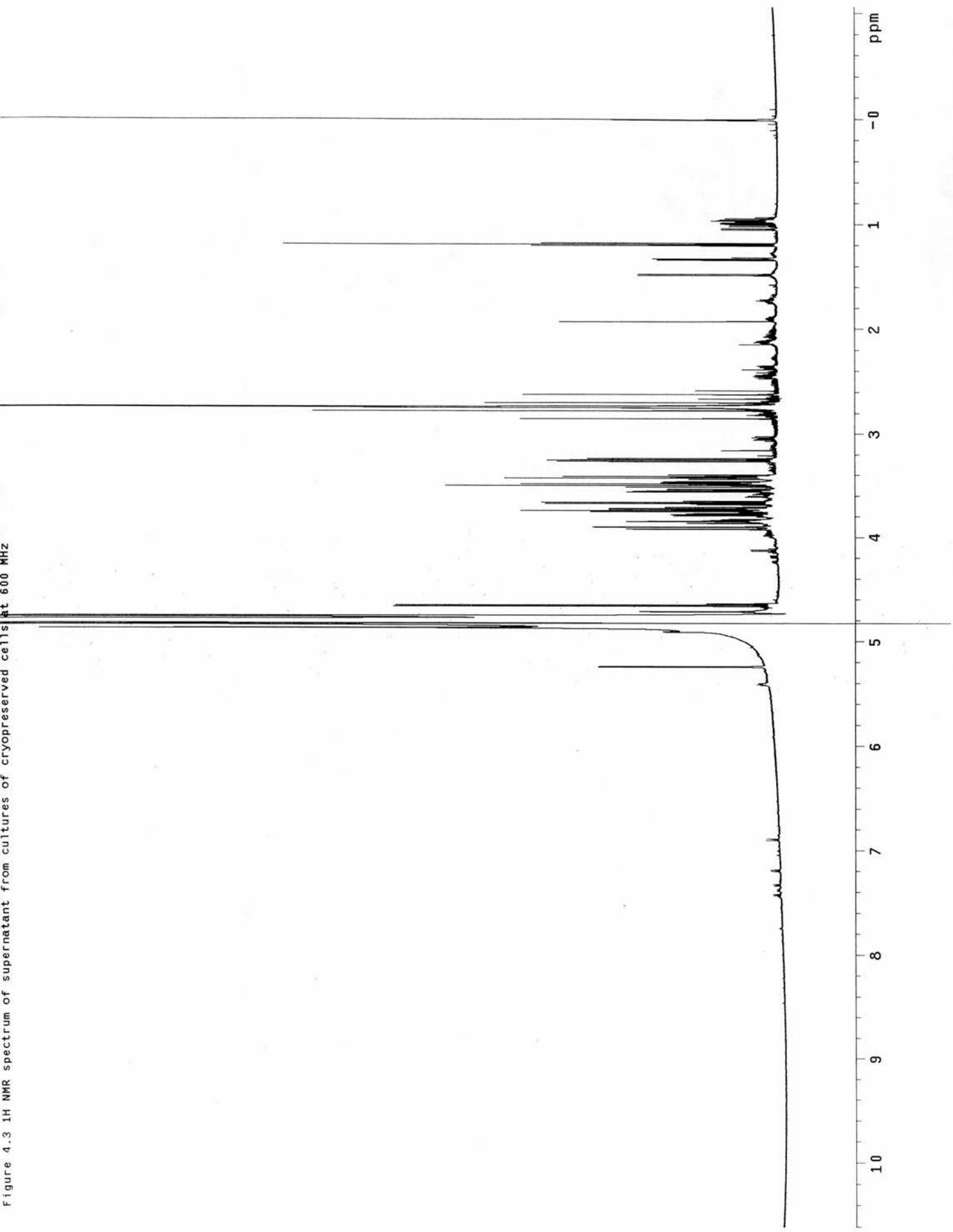
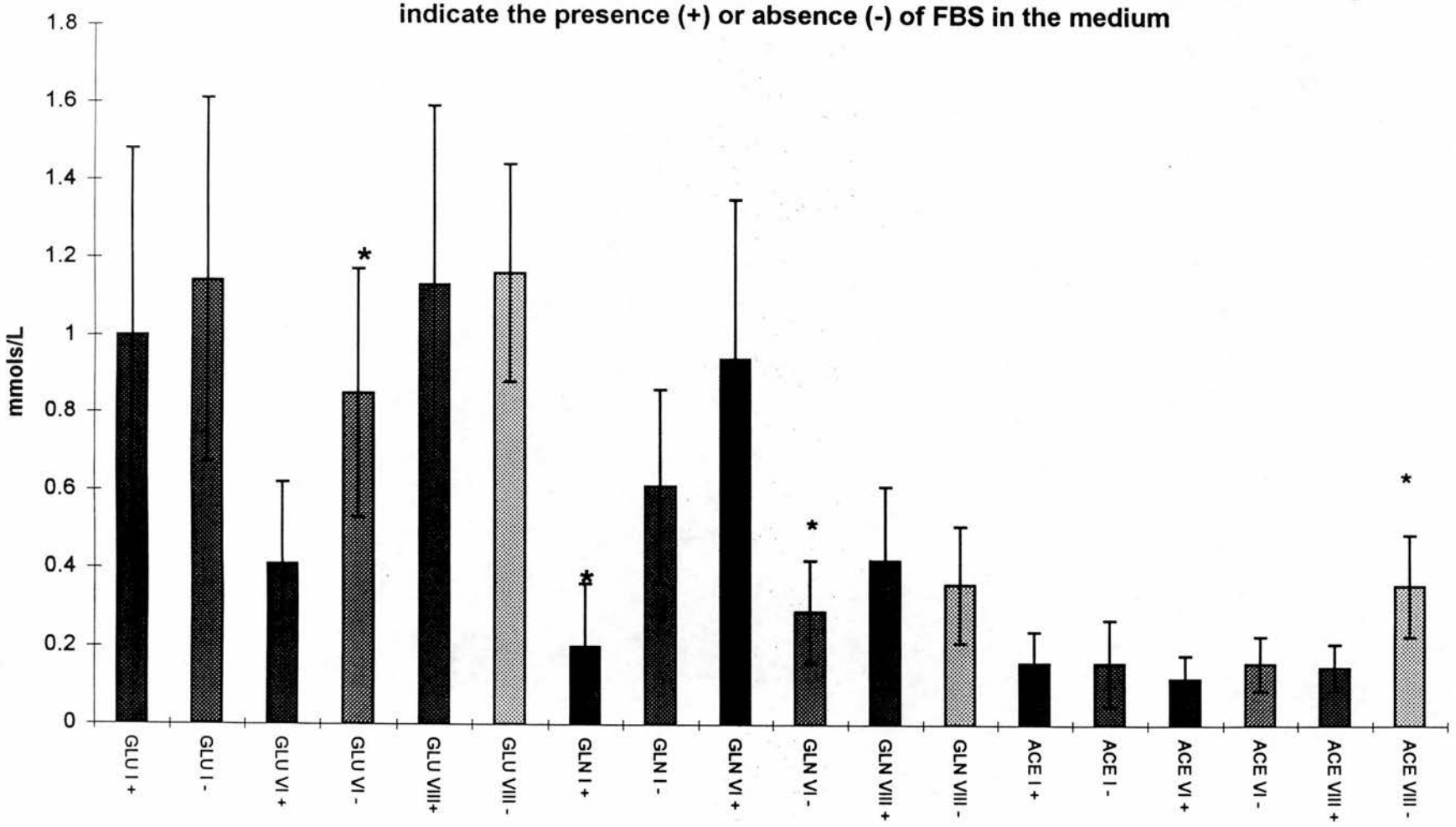


Figure 4.3 1H NMR spectrum of supernatant from cultures of cryopreserved cells at 600 MHz

Figure 4.4

Comparison between concentrations of Glutamate (GLU), Glutamine (GLN) and Acetate (ACE) in supernatants of cryopreserved hepatocytes by Protocols I (I),VI (VI) and VIII (VIII). The + or - signs indicate the presence (+) or absence (-) of FBS in the medium



Results shown are means and error bars are SEM. Results are compared with same protocol *p<0.05

TABLE 4.1

Comparison of viability and plating efficiency in cells cryopreserved with or without Foetal Bovine Serum using different hepatocyte cryopreservation protocols. . Abbreviations are as follows: Protocol I (I), Protocol VI (VI), Protocol VIII (VIII), Cultures with foetal bovine serum (+ FBS), Cultures without foetal bovine serum (- FBS)

	I +FBS	I-FBS	VI+FBS	VI -FBS	VIII+FBS	VIII-FBS
Viability	40±5.1%	61±3.8%*	43±7.3%	56±6.9%	43±5.0%	52±4.1%
Plat. Eff.	12±2. 3%	16±3.1%	10±0.9%	15±3.3%	9±1.2%	13±2%

Results shown are mean ± SEM. * $p<0.05$ compared to measurements from the same protocol.

4.4.5 Effect of different culture media on activity of plated hepatocytes

Four different isolations were assessed. William's E and 199 media were assessed. No significant differences were observed either between cryopreservation protocols or between different incubation and preservation media. There was a trend for less lactate production and less glucose uptake in cells incubated with William's E medium but the results were not significant. All further experiments were conducted using William's E medium as incubation and cryopreservation medium.

4.4.6 Effect of collagen coating of culture plates

Four different isolations were assessed. Both protocols used for the cryopreservation of plated hepatocytes were assessed. No significant differences were observed either between cells on standard cell culture dishes and collagen coated cell

culture dishes, or between protocols. All further experiments were conducted using standard cell culture dishes.

4.4.7 Comparison between different cryopreservation protocols

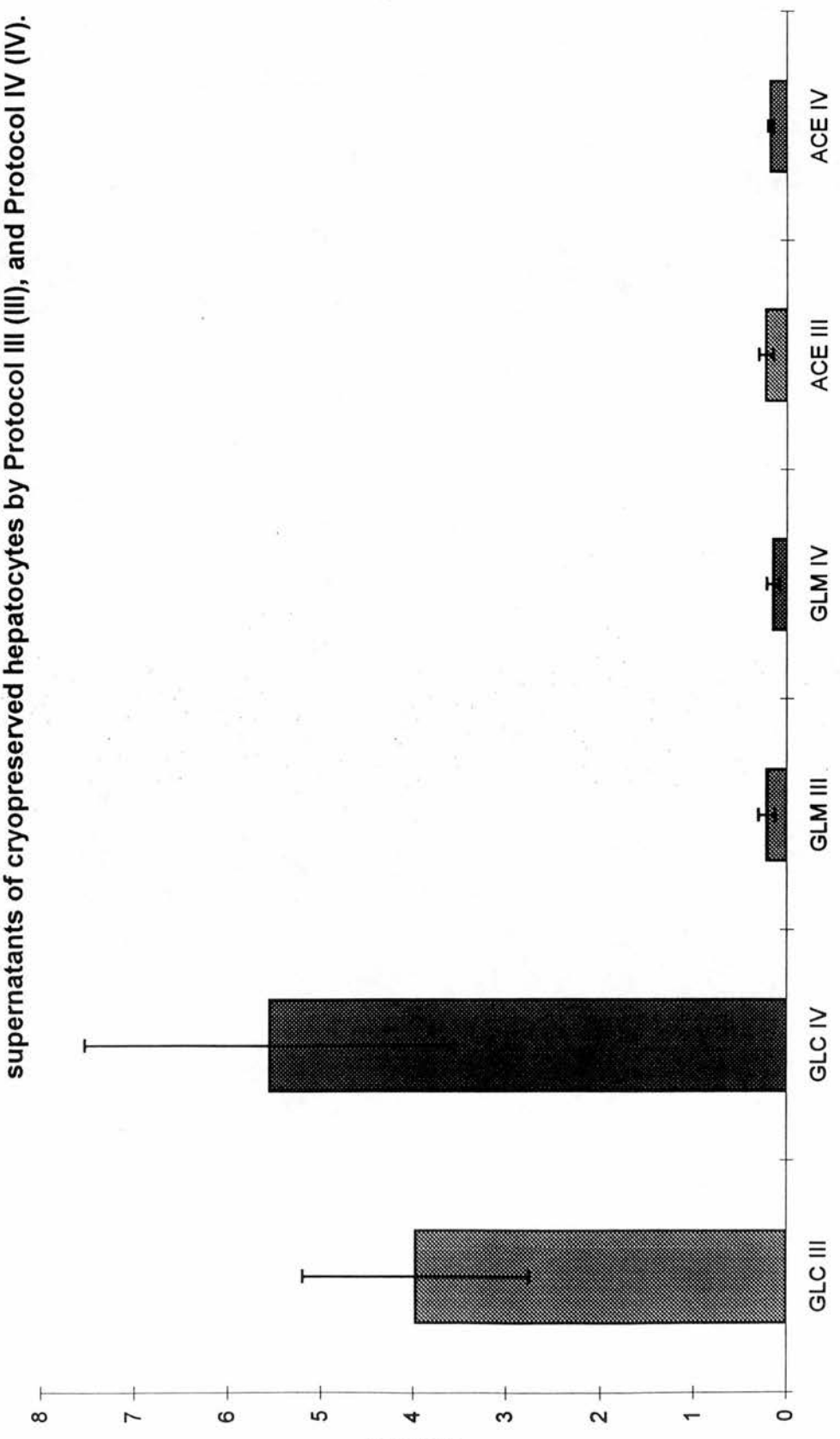
Protocols I and II (quick freezing protocols) were compared. Five different isolations were assessed. No significant differences were observed overall between the two protocols.

Protocols III and IV (slow freezing protocols) were compared. Figure 4.5 summarises the results of these experiments (n=5). No significant differences were observed overall between the two protocols. There was a trend for more acetate production and less glutamine uptake by the cells using protocol III but the results were not statistically significant.

Protocols V, VI and VII (stepwise freezing protocols) were compared. Five different isolations were assessed. Figure 4.6 summarises the results of these experiments (n=5). No significant differences were observed overall between the three protocols. There was a trend for cells cryopreserved by protocol VI to consume more glucose but the results did not reach statistical significance. There was a trend for cells cryopreserved by protocol VI to produce less acetate but the results did not reach statistical significance. There was a trend for cells cryopreserved by protocol VII to produce less lactate but the results did not reach statistical significance.

Figure 4.5

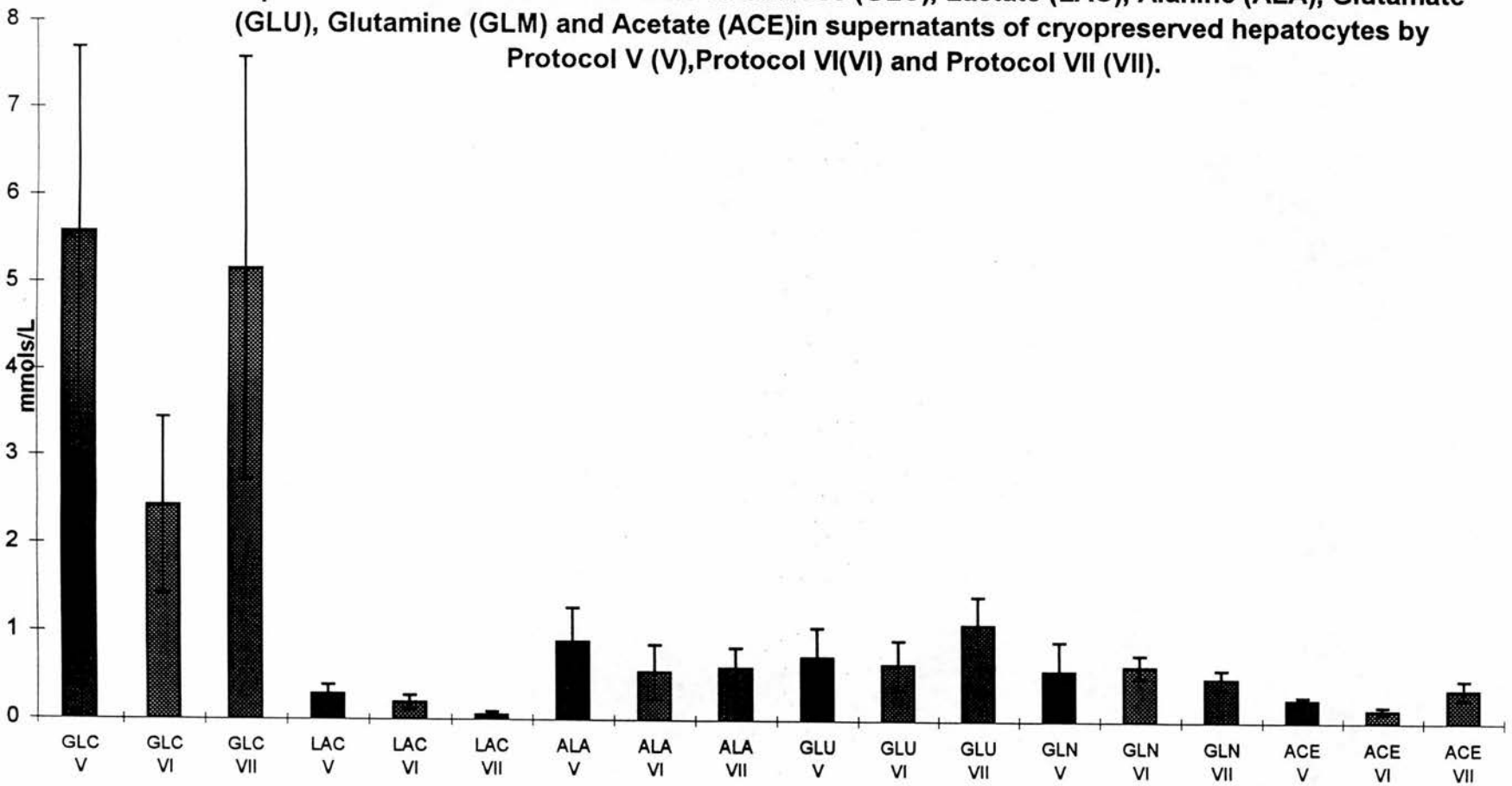
Comparisons between concentrations of Glucose (GLC), Glutamine (GLM) and Acetate (ACE)in supernatants of cryopreserved hepatocytes by Protocol III (III), and Protocol IV (IV).



Results shown are means and error bars are SEM.

Figure 4.6

Comparisons between concentrations of Glucose (GLC), Lactate (LAC), Alanine (ALA), Glutamate (GLU), Glutamine (GLM) and Acetate (ACE)in supernatants of cryopreserved hepatocytes by Protocol V (V),Protocol VI(VI) and Protocol VII (VII).



Results shown are means and error bars are SEM.

4.4.8 Comparison between cryopreserved and freshly isolated hepatocytes

To compare the metabolic activity of cryopreserved hepatocytes (CP) and freshly isolated hepatocytes (FH) all assessed protocols were used. Quick freezing (QF) protocols (I and II) were combined together as were protocols III and IV (slow freezing, SF) V, VI and VII (stepwise freezing protocols, STF) and protocols VIII and IX (stepwise freezing of plated hepatocytes, PH), as no significant differences between were observed. Standard experimental conditions comprised cell suspended in William's E medium without FBS supplementation, and plated hepatocytes were plated on standard cell culture dishes.

Experiments were conducted during ten different isolations. The viability post thaw was significantly reduced in CP cells compared to FH cells ($p<0.05$ in all measurements). Plating efficiency was also significantly reduced in cryopreserved hepatocytes ($p<0.01$ in all measurements) (Table 4.2).

TABLE 4.2

Comparisons of viability and plating efficiency between cells cryopreserved under different protocols and freshly isolated cells. Abbreviations are as follows: Quick freezing protocols (QF), Slow freezing protocols (SF), Stepwise freezing protocols (STF), cells frozen on plates (PH), freshly isolated cells (FH)

	FH	QF	SF	STF	PH
Viability	82±9.4%	56±8.8%*	53±7.6%*	55±8.3%*	62±5%*
Plat.eff.	87±4.3%	13±2.6%**	15±3.6%**	9±2.1%**	N/A

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to the measurements from fresahly isolated hepatocytes.

4.4.8.1 Glycolysis and gluconeogenesis

Table 4.3 summarises the results of glucose metabolism. All CP cells and FH cells consume glucose for their energy needs. No significant differences were observed between CP cells and FH cells. SF hepatocytes consumed more glucose but the results were not significant. Lactate production was apparent by all cells. FH cells produced significantly more lactate then CP cells ($p<0.0000$ in all measurements). By contrast, pyruvate, which is a normal constituent of William's E medium, was taken up from the culture medium by all cells. The uptake was significantly less in FH cells compared to all cryopreservation protocols (FH vs QF($p<0.01$), FH vs SF ($p<0.016$), FH vs STF ($p<0.0073$), FH vs PH ($p<0.009$)). Alanine, a central amino acid involved in many metabolic pathways had different fates depending on the protocol. There was a small overall production of alanine in PH and QF hepatocytes and a small overall uptake in SF and STF hepatocytes. In FH cells there was a net consumption of alanine and this was statistically significant compared with all cryopreservation protocols (FH vs QF($p<0.0068$), FH vs SF ($p<0.0084$), FH vs STF ($p<0.018$), FH vs PH ($p<0.0071$)). There was a net consumption of threonine overall by QF, PH and FH cells, the biggest consumption occurring in FH cells. By contrast, SF and STF cells exhibited a small production of threonine that was reflected by an increase in the concentration of threonine in culture supernatants.

TABLE 4.3

Comparison of glucose metabolism between cells cryopreserved under different protocols and freshly isolated cells. Abbreviations as in Table 4.2 plus William's E (Will. E)

	Will. E	FH	QF	SF	STF	PH
Glucose	7.7	5.51±0.76	5.02±0.48	4.57±0.51	5.52±0.68	5.95±0.78
Lactate	0	3.35±0.90	0.15±0.06**	0.22±0.06**	0.12±0.05**	0.18±0.03**
Pyruvate	0.2	0.12±0.04	0.03±0.01**	0.04±0.00*	0.02±0.00**	0.03±0.00**
Alanine	0.71	0.21±0.06	0.78±0.08**	0.66±0.0**	0.60±0.17*	0.76±0.12*
Threonine	0.23	0.11±0.06	0.16±0.10	0.20±0.04	0.25±0.06	0.13±0.02

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to baseline values. Absolute values are in mmols/L

4.4.8.2 Urea and glutamine synthesis

Table 4.4 summarises the results for glutamate, glutamine histidine and arginine metabolism. The pattern observed in all supernatants was of glutamine uptake, accompanied by glutamate release in the medium. This represented intact transamination activity. Glutamine can also be used in DNA synthesis. Glutamine uptake by FH cells was significantly less compered to CP cells and reached statstical significance in all measurements (FH vs QF($p<0.005$), FH vs SF ($p<0.0071$), FH vs STF ($p<0.025$), FH vs PH ($p<0.0071$)). No significant differences were observed concerning the release of glutamate back into the medium. Complete consumption of

histidine was observed in all supernatants. Arginine uptake was also comparable from all supernatants.

TABLE 4.4

Comparison between concentrations of glutamate synthesis precursors arginine and histidine, glutamate and glutamine between cells cryopreserved under different protocols and freshly isolated cells. Abbreviations as in Table 4.3.

	WE	FH	QF	SF	STF	PH
Histidine	0.07	0.02±0.01	0	0	0	0
Arginine	0.28	0.13±0.06	0.12±0.05	0.11±0.04	0.15±0.05	0.13±0.05
Glutamate	0.21	0.86±0.10	0.86±0.14	0.86±0.13	0.71±0.13	0.81±0.11
Glutamine	1.4	0.83±0.13	0.21±0.10**	0.27±0.08**	0.48±0.15*	0.27±0.08**

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to baseline values. Absolute values are in mmols/L

4.4.8.3 Acetyl-CoA precursors and acetate.

Table 4.5 summarises the results for leucine, phenylalanine and tyrosine metabolism which are some of the precursors for acetyl CoA and acetate production. There was net uptake of leucine, from the medium by all cells. Phenylalanine was consumed mostly by FH cells. There was a smaller consumption of phenylalanine by CP cells. The results reached statistical significance if we compared FH vs QF ($p<0.025$ and FH vs STF ($p<0.032$). Tyrosine was preferentially consumed by FH

cells. The results were statistically significant compared with all cryopreservation protocols (FH vs QF($p<0.033$), FH vs SF ($p<0.042$), FH vs STF ($p<0.029$), FH vs PH ($p<0.02$)). Overall minimal amounts of tyrosine were consumed by CP cells. Acetate production was significantly higher in FH cells compared to CP cells ($p<0.0000$ in all measurements).

TABLE 4.5

Comparison between concentrations of acetyl-CoA precursors leucine, phenylalanine and tyrosine and acetate, between cells cryopreserved under different protocols and freshly isolated cells. Abbreviations as in Table 4.3.

	Will. E	FH	QF	SF	STF	PH
Leucine	0.4	0.21±0.03	0.31±0.05	0.26±0.02	0.20±0.04	0.33±0.04
Phenylal.	0.14	0.06±0.01	0.14±0.03*	0.11±0.05	0.13±0.07*	0.09±0.04
Tyrosine	0.19	0.07±0.02	0.19±0.06*	0.15±0.03*	0.18±0.03*	0.14±0.04*
Acetate	0	7.82±2.00**	0.19±0.05**	0.23±0.03**	0.14±0.04**	0.15±0.03**

Results shown are mean ± SEM. * $p<0.05$, ** $p<0.01$ compared to baseline values. Absolute values are in mmols/L

4.4.8.4 Succinate precursors.

Figure 4.7 summarises the results for valine, isoleucine and methionine metabolism which are the precursors for succinate formation that would then feed Kreb's cycle. A small net consumption of valine by all cells, was observed. Isoleucine uptake was very small from most cell supernatants but the supernatants from SF cells showed no net isoleucine consumption.. The fate of methionine which is a constituent substance of the William's E medium seems to be similar in all cultures. There was no significant uptake of methionine by any culture system.

4.4.8.5 ETHANOL

Figure 4.8 summarises the results for ethanol concentrations. Ethanol was identified in all culture supernatants. The ethanol concentration was elevated in all supernatants. It was markedly elevated in PH and FH cells , that is in all cell cultures which have reached confluence at the time of the experiments

Figure 4.7

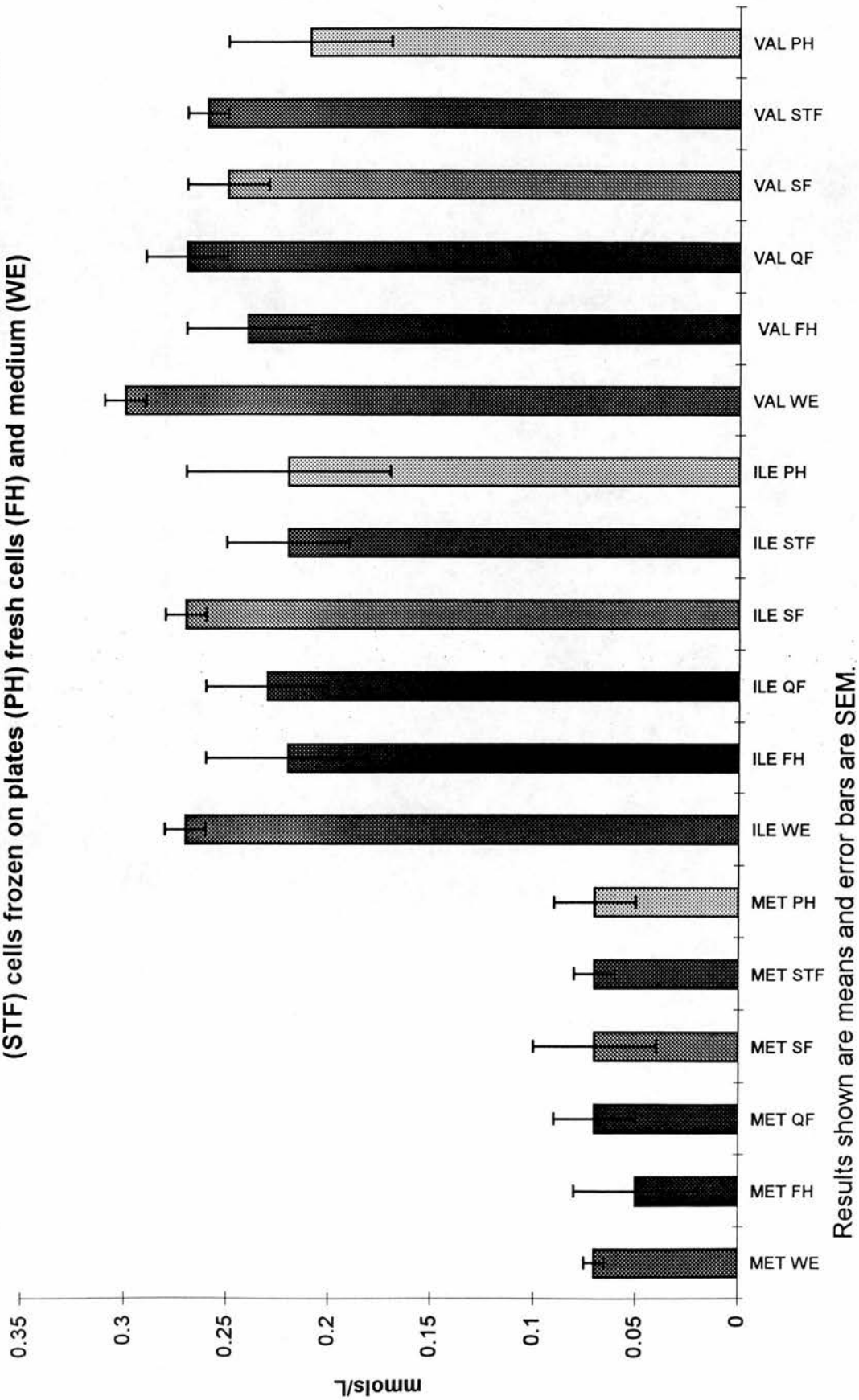
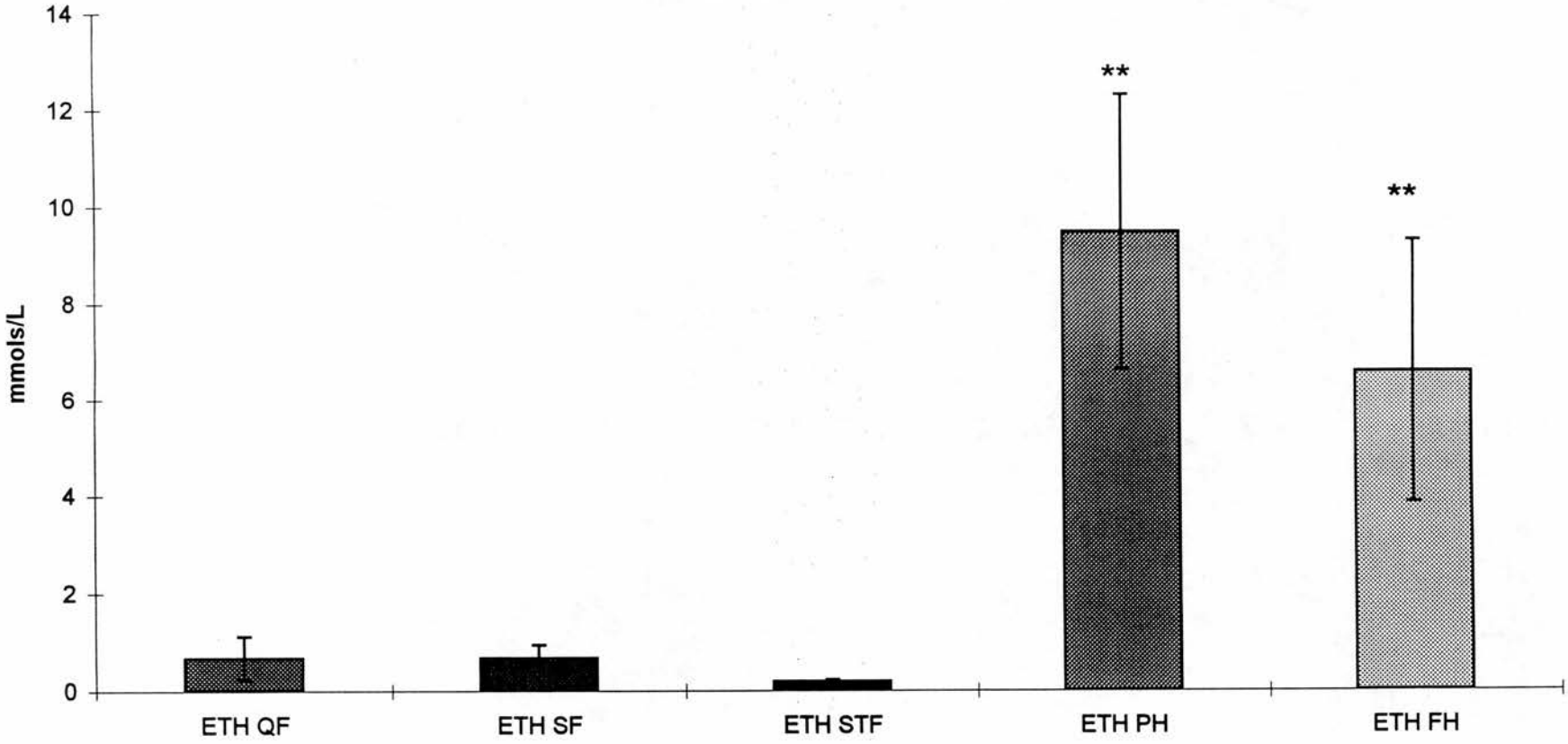

Comparisons between concentrations of Valine (VAL) Isoleucine (ISO) and Methionine (MET) in supernatants from cell cryopreserved by Quick Freezing (QF) Slow Freezing (SF) Stepwise Freezing (STF) cells frozen on plates (PH) fresh cells (FH) and medium (WE)
mmols/L
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
VAL PH
VAL STF
VAL SF
VAL QF
VAL FH
VAL WE
ILE PH
ILE STF
ILE SF
ILE QF
ILE FH
ILE WE
MET PH
MET STF
MET SF
MET QF
MET FH
MET WE
Results shown are means and error bars are SEM.

Figure 4.8

Comparison between concentrations of Ethanol (ETH) in cell supernatants from different cryopreservation protocols. Protocol abbreviations as per Figure 4.7.



Results shown are means and error bars are SEM.
**p<0.01

4.5 DISCUSSION

This study was undertaken to assess the metabolic functions of cryopreserved primary porcine hepatocytes with the help of ^{1}H NMR Spectroscopy. These cells were kept in cryogenic storage in a LN_2 atmosphere for 1 week, before being plated. The culture supernatants were examined at 24 h after plating.

In our study the viability of the CP cells was examined and was found to be significantly less than that of FH cells. There was a trend for better viability for plated hepatocytes but was inferior to that of FH cells. It is generally thought that hepatocytes are very difficult cells to cryopreserve as they seem to be very sensitive to intracellular ice formation (Darr et al,1997) and the known cytoprotectants are not of much help (Toshchakov et al,1991). This is also reflected by the extremely poor plating efficiency of the cells preserved in suspensions and by the inability of the hepatocytes to attach to microcarriers properly.

In this study we examined some of the major metabolic pathways of the CP hepatocytes and compared them to FH hepatocytes. We are going to examine those pathways in detail below.

Glucose from the glucose rich William's E medium was consumed by both CP cells and FH cells. No significant differences were observed either between different cryopreservation protocols or between CP and FH cells. CP cells seemed to have a less active glycolytic pathway but the net production of alanine from QF and PH cells was an indication that pyruvate was transformed to alanine and glycolysis did occur. There was a very small production of lactate and most of the pyruvate was consumed by CP cells (Denton et al, 1978). The conversion of pyruvate to alanine suggets that

there was definitely no active gluconeogenesis present. Results on threonine concentration were different depending on the cryopreservation protocol used. PH and QF hepatocytes seemed to consume threonine and by converting it to alanine. Those results are in agreement with inactive gluconeogenesis and high alanine concentrations in supernatants of PH and QF cells. There was a negligible uptake of threonine by SF hepatocytes and there was a net production of threonine in STF cells supernatants from protein breakdown, which was not directly converted to alanine.

By contrast FH cells seemed to have a more active glycolysis. Although the abundant lactate production could be a sign of anaerobic metabolism the fact that alanine was consumed in big quantities proves that pyruvate synthesis was present and gluconeogenesis was active (Seglen et al, 1974). There was also a significant threonine uptake which implied that threonine was converted to alanine.

Successful turnaround of nitrogen compounds is another key function of the hepatocytes. This is reflected in transamination reactions and urea production through the urea cycle. The central player in transamination reactions is of course glutamate which accepts the nitroso radical of α-ketoacids during their catabolism (Fuchs et al, 1994). Net production of glutamate was observed in all culture supernatants in CP and FH hepatocytes. Glutamine is produced from glutamate when need for scavanging of ammonium cations is present. It is then used as a nitrogen donor in the synthesis of purine and pyrimidine bases and hence nucleic acid synthesis (Lang et al,1990). Glutamate and glutamine are also a useful buffer in acidotic conditions (Gerlach et al, 1996). Significantly more glutamine was consumed by CP cells than by FH cells. It is reasonable to conclude then that transamination was active in CP cells.

Arginine uptake from the media by both CP and FI cells showed that arginine was introduced into the urea cycle. All supernatants from cryopreserved cells showed that histidine from the medium was entirely consumed by the hepatocytes. Histidine uptake is indicative of an ability of hydroxylation and further transformation of histidine into glutamate can take place in hepatocytes (De Blaaw et al, 1999). Our results confirm that transamination occurs and the urea cycle is active in CP cells in accordance with previous reports (Lawrence et al, 1991; Kasai et al 1993).

Acetate production from the fatty acids via ketogenesis is characteristic of the hepatocyte (Seifter et al, 1994). Significantly less acetate was produced by the CP cells indicating a dysfunctioning fatty acid oxidation and ketogenesis. In comparison FH cells produced and exported significant amounts of acetate. Although studies have shown defective fatty acid oxidation in new born piglets (Duee et al, 1994), the fact that FH cells produced acetate confirms that there is no enzyme deficiency in our piglet population. Looking at ketogenic amino acids leucine was preferentially taken up by both CP and FH cells. The other ketogenic amino acids isoleucine, phenylalanine and tyrosine showed a small and comparable uptake by CP cells cryopreserved by all methods. FH cells consumed more isoleucine, phenylalanine and tyrosine and this represents evidence of more active ketogenesis by FH cells.

Some of succinate precursors' fate was monitored during our experiments. We mentioned previously the small uptake of isoleucine. A similar pattern was observed by valine as all hepatocytes utilised very little valine and there was no difference between FH and CP cells. The pattern for methionine was of no net methionine consumption overall by any CP cells. There was a 30% reduction of methionine concentration in the supernatants of FH cells. Overall those results indicated that

there was no significant succinate production to feed the Kreb's cycle and this is in agreement with our hypothesis that Kreb's cycle activity is impaired.

The liver has a very limited capacity to metabolise branch chain amino acids (BCAAs) as it lacks almost completely the necessary enzymes to metabolise leucine to a-ketoisocaproate, isoleucine to a-ketoß-methylvalerate and valine to a-ketoisovalerate in basal conditions. This is true in *in vivo* situations though and in the well fed state. It is possible that hepatocytes under stress as they possess the mechanism for production of the necessary enzymes, they would try and utilise BCAAs to produce necessary proteins (Fuchs et al, 1994). Our results showed that CP and FH cells preferentially utilised leucine for protein synthesis or transamination reactions which is the most abundant BCAA in William's E medium. There was almost negligible uptake of isoleucine and valine by all cell cultures. The fact that all BCAA's use the same transporter to enter the cells could account for that observation as cells try to restore an equilibrium in the extracellular milieu.

Aromatic amino acids present in the culture medium were also taken up by the hepatocytes. CP cells almost exclusively took up histidine from the medium and little tyrosine or phenylalanine. FH cells also took up histidine but tyrosine and phenylalanine as well. Phenylalanine can be converted to tyrosine and lead to the formation of ketoacids.

High levels of ethanol were observed in all culture supernatants. Their presence is enigmatic. The obvious theory of contamination by bacteria or fungi is confounded by the sterility of media on conventional testing. The fact that we used ethanol in our laboratory as a decontaminant on incubators and laminar flow hoods was also tested by introducing alcohol free isolations. Cell culture supernatants

though from those isolations clearly showed evidence of ethanol in the cell culture supernatant. These observations have previously been reported in biological fluids of animals (Nicholson et al., 1985; Gosden et al,1990; Foxall et al. 1992) and one plausible explanation is that some mammalian species under stress conditions have the ability to produce ethanol under conditions of alcohol dehydrogenase inhibition

We were surprised to find that supplementation of the cryopreservation medium with FBS, was actually detrimental to the cells. Our results confirm the observation that FBS made no difference in the functional status of the hepatocytes (Lawrence et al, 1993). We have also observed that different media and the coating of plates with collagen did not make any significant difference in accordance with previous studies (Watts et al, 1996.).

Overall, our results have shown that ^{1}H NMR Spectroscopy at 600MHz is an accurate tool to monitor the metabolic activity of cryopreserved and freshly isolated cultured cells. They would be in agreement with the hypothesis that although cryopreserved hepatocytes are metabolically active, major metabolic pathways are dysfunctioning as cryogenic storage could significantly impair the function of temperature sensitive metabolic enzymes. Although cryopreserved cell have been used as the biological component of BALSS that have recently gone into clinical trials it is clear that CP cells do not perform as well as FI cells. The fact that these systems can be performing as only a biological adsorbent detoxifying substances that could be responsible for the acute liver failure has been proposed (Flendrig et al, 1997) and merits further investigation.

Our results have shown that the up to date cryopreservation protocols do produce similar results. The fact that new biological cytoprotectants will be on the

market shortly would guarantee further investigations. Further efforts will go into the understanding of the freezing process itself in order to optimise cryopreservation and to make it a valuable tool for the development for liver support systems in the future.

CHAPTER 5

STUDIES ON HYPOTHERMICALLY PRESERVED HEPATOCYTES

5.1 INTRODUCTION

The complex physiological role of the liver and its pivotal role in a diversity of biochemical pathways is well recognised. Methods to isolate the liver and to study its metabolic functions in an environment free from the regulatory signals it receives in vivo have been sought since the early days of modern biomedical research. The introduction of the two step collagenase perfusion technique for the isolation of liver parenchymal cells (Barry et al, 1969) has been a major breakthrough for the study of a variety of liver functions. Extensive progress has been made since, in our ability to isolate and maintain primary hepatocytes in monolayer cultures for several days (Gerlach et al, 1994; Sielaff et al,1995; Koebe et al, 1996; Jauregui et al, 1997).

Primary hepatocytes are widely used in *in vitro* models of toxicology and pharmacology. The metabolism of xenobiotics by primary hepatocytes has been extensively investigated and has been used in recent years to help produce safer drugs by studying drug metabolism and to reduce the number of *in vivo* animal toxicology studies (Strom et al, 1983; Butterworth et al, 1989; Guillouzo et al, 1997).

Increasing demand for isolated hepatocytes has led scientists to experiment with different animal species. Rat hepatocytes are extensively used for in vitro studies in toxicology and pharmacology (Paine et al, 1982; Ratanavasanh et al, 1988). Although primary porcine hepatocytes now seem to be the cells of choice in liver support systems, the use of human hepatocytes in those systems when treating

patients has been advocated (Jauregui et al, 1997; Demetriou et al, 1998b; Clement et al, 1998).

In the previous chapter we have looked at cryopreserved hepatocytes and their metabolic functions. Overall our results suggest that key metabolic pathways of cryopreserved hepatocytes are impaired and that cryopreserved hepatocytes are not ideal for use in BALSS and in hepatocyte transpplantation.

Hypothermic preservation of primary hepatocytes has been suggested as a means of temporary liver cell storage (Marsh et al, 1991; Poullain et al, 1992; Vara et al, 1995; Oesch et al, 1995; Sakai et al, 1996; Platt at al, 1996). Such storage is important if BALSS are to be commercially developed. Hypothermic storage can affect hepatocyte viability and plating efficiency (Chesne et al, 1993b; Naik et al, 1997). So far studies evaluating the metabolic functions of hypothermically preserved hepatocytes have been limited to monitoring enzyme activities, (Marsh et al, 1991; Poullain et al, 1992; Vara et al, 1995; Sakai et al, 1996; Platt at al, 1996) drug and xenobiotic metabolism (Poullain et al, 1992; Oesch et al,1995; Sakai et al, 1996; Platt at al, 1996) and albumin production (Marsh et al,1991; Poullain et al,1992; Vara et al,1995; Sakai et al,1996). This is because the techniques to measure the production of substrates from the different biochemical cycles occurring within the hepatocytes are not widely available.

The aim of this study was to validate the use of NMR Spectroscopy as a tool to assess metabolic functions of primary porcine hepatocytes and to monitor major metabolic pathways in those cells during culture following hypothermic preservation

5.2 MATERIALS AND METHODS

Hepatocyte isolation was performed as previously described in Chapter 3.

5.2.1 Hypothermic cell preparation

Our hypothermic cell preparation is based on that described by Poullain et al,(1992). Freshly isolated primary porcine hepatocyte suspensions in Williams E medium were centrifuged (25 g for 5 min) and resuspended in Leibovitz L-15 medium supplemented with antibiotics (penicillin 50 mg /ml, streptomycin 10mg/ml and gentamicin 0.1 mg/ml). Hepatocytes (10^7 /ml) were stored at 4°C in sealed 3 ml Nunc Cryovials (Nunc, Denmark) for 48 h under an air atmosphere. At 48 h hepatocytes were gently resuspended and cell viability was assessed by the trypan blue exclusion test and plating efficiency by percentage LDH activity. Cells were then centrifuged (25 g for 5 min) washed in Hank's Balanced Salt Solution (HBSS) buffer and then resuspended in Williams E medium before seeding.

5.2.2 Cell culture

Both fresh and hypothermically preserved hepatocytes were seeded at a density of $8x10^6$ cells / plate (90 mm standard cell culture dishes) (Corning Inc.). The culture medium used was a supplemented Williams E medium. Supplements included penicillin, streptomycin, gentamicin, amphoterocin B, porcine insulin, L-glutamine, dexamethasone and Long Epidermal Growth Factor (L-EGF). The culture medium was renewed every day during the culture period. Cultures were maintained under an atmosphere of 95% air and 5% CO_2 at 37 °C. They were routinely examined using phase contrast microscopy before every culture medium renewal.

5.2.3 NMR Spectroscopy

NMR Spectroscopy monitoring was performed as discussed in Chapter 2. Sample preparation, data acquisition and quantitation of compound concentrations were performed as discussed in Chapter 2.

5.2.4 Statistical analysis

Comparisons between freshly isolated (FI) and hypothermically preserved (HP) hepatocytes from the same isolation were made. The values of each experiment were the mean of six cultures. Results are expressed as mean ± standard error of the mean.

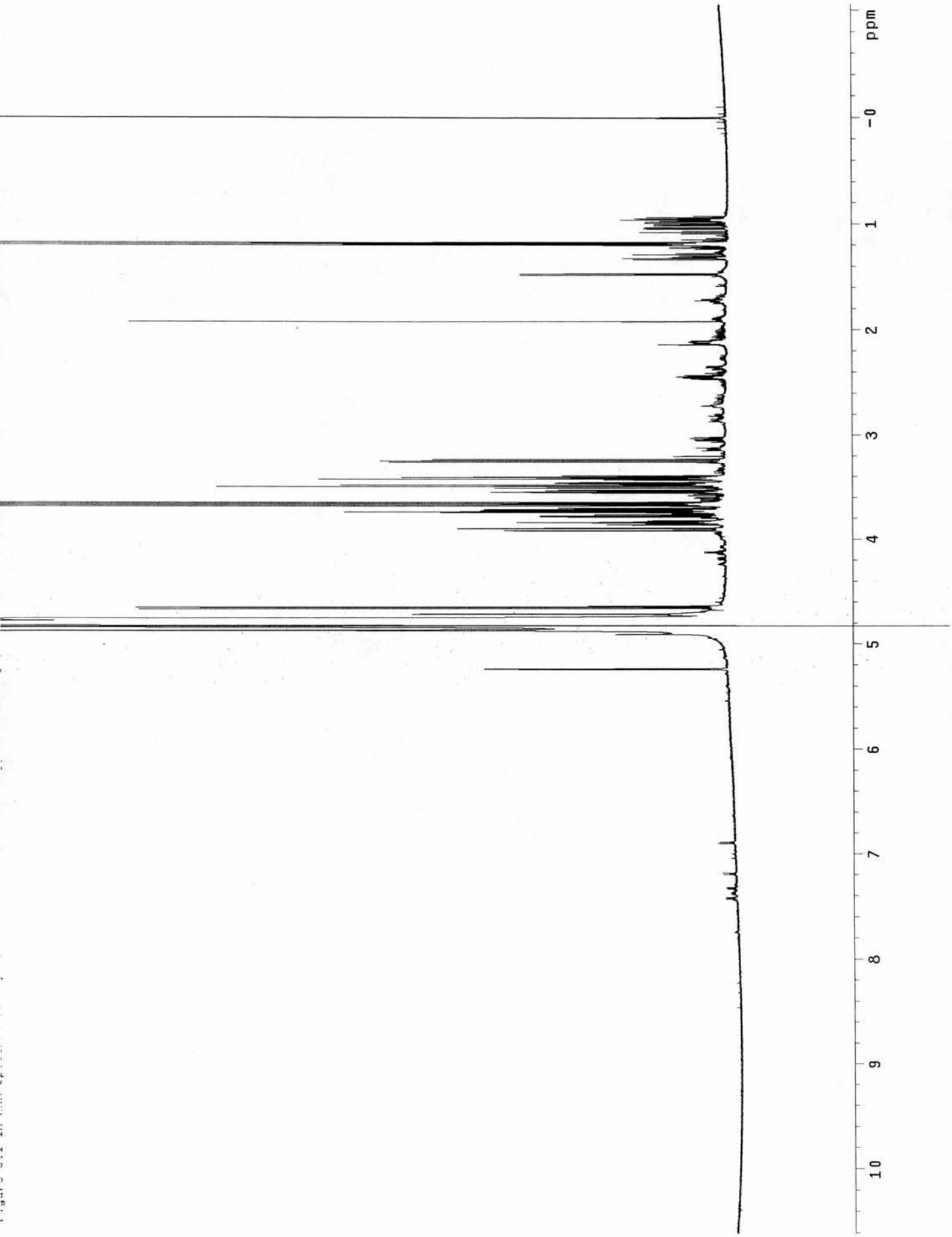
5.3 RESULTS

Results are expressed as mmols/L. Six different primary porcine hepatocyte isolations were evaluated. Viability was 78.1 ± 1.2% at isolation, 69 ± 3.4% at 24 hours and 58.9 ± 3.8% at 48hours of hypothermia. Plating efficiency was 87 ± 4% for freshly isolated (FI) cells and 33.6 ± 7.6% for hypothermically preserved (HP) cells at 48h.

Both HP and FI hepatocytes were observed using phase contrast microscopes. There were no morphological differences between the two groups of hepatocytes.

5.3.1 Quantitative NMR data

Figure 5.1 shows single pulse 600 MHz ^{1}H NMR spectra of supenatants from cultured HP hepatocytes 48 h after plating. Peaks were assigned to low

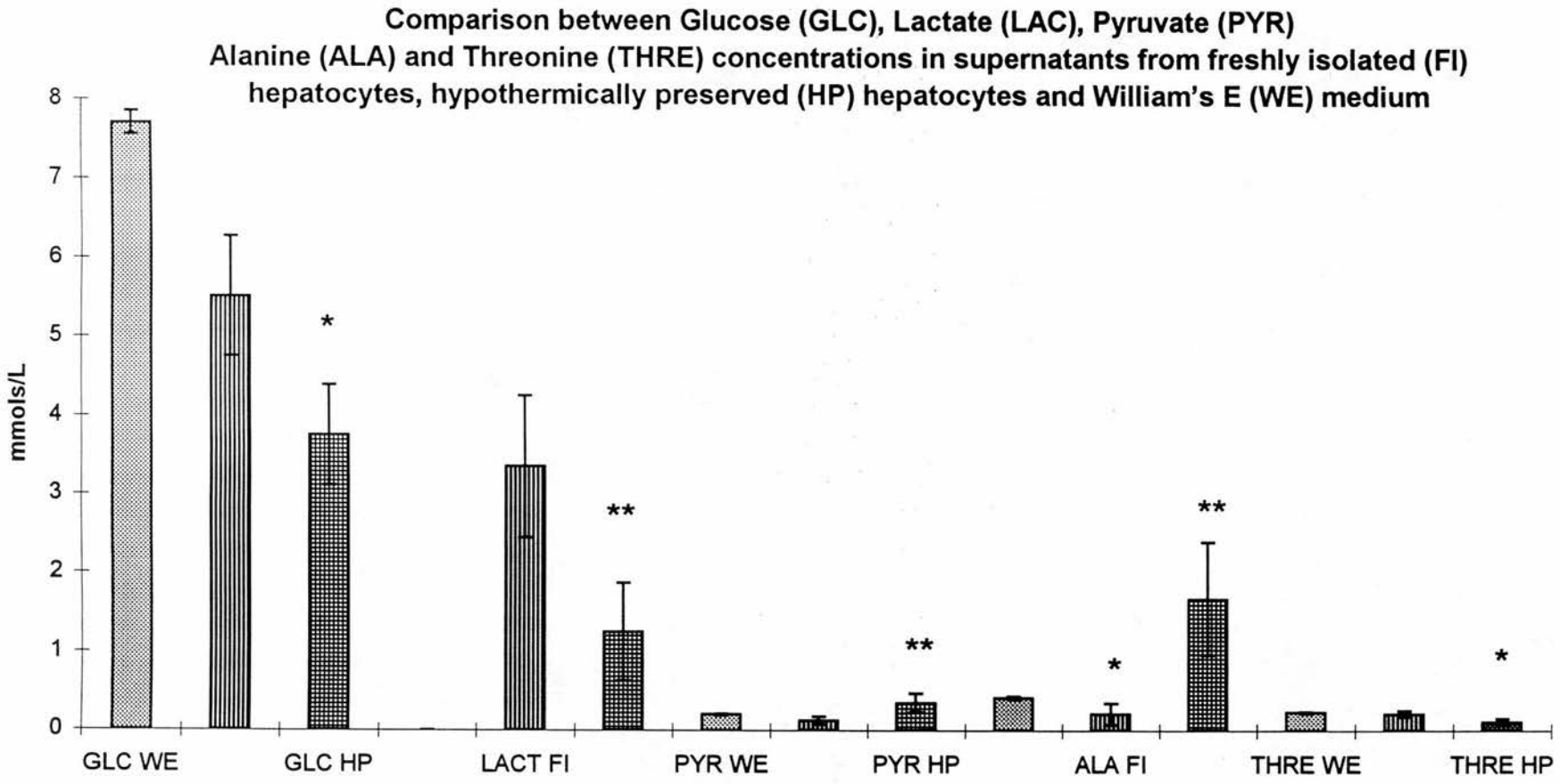

ppm
-0
1
2
3
4
5
6
7
8
9
10

5.3.2 Glycolysis and gluconeogenesis

Figure 5.2 shows the levels of glucose, lactate, pyruvate alanine and threonine in supernatants of FI cells, HP cells, and supplemented William's E medium for comparison.

The results showed that there was a net consumption of glucose in both FI and HP cell supernatants. If we compared glucose consumption by FI and HP cells there was significantly more glucose consumed by HP cells ($p<0.038$) . Lactate was produced by both HP and FI cells but production of lactate by FI cells was significantly higher than production by HP cells ($p<0.0076$). Examination of pyruvate which is normally a constituent of William's E medium, enabled us to conclude that there was a consumption of pyruvate by FI cells and a net production of pyruvate by HP cells. Pyruvate concentration in HP cells supernatants was significantly higher than in supernatants on FI cell cultures ($p<0.01$). Alanine, a central amino acid, involved in many pathways is gluconeogenic and can also be converted to pyruvate. We observed a significant reduction of alanine concentration in the supernatants of FI cells compared to William's E medium ($p<0.046$), but noted that there was a significant increase in the alanine concentration in the supernatants of HP cells compared to William's E medium ($p<0.009$). If we look at threonine which is an amino acid used by hepatocytes as a substrate for alanine synthesis we observed that FI cells consumed very little threonine but there was significant reduction of threonine concentration in supernatants of HP cells ($p<0.04$).

Figure 5.2



Results shown are means and error bars are SEM.
Comparisons on the figure are made with previous bar.
*p<0.05 ** p<0.01

5.3.3 Urea and glutamine synthesis

Figure 5.3 shows the levels of glutamine , glutamate, arginine and histidine in supernatants of FI cells, HP cells and supplemented William's E medium for comparison.

Glutamine uptake was accompanied by glutamate release in both supernatants. Glutamine uptake in both FI and HP supernatants was comparable. Glutamate release was also comparable. This reflects intact transamination capacity by both culture modalities. Arginine and histidine uptake was significant in both supernatants ($p<0.032$ for arginine and $p<0.028$ for histidine). The amounts consumed were comparable for both supernatants, which indicates urea cycle activity.

5.3.4 Acetyl-CoA precursors

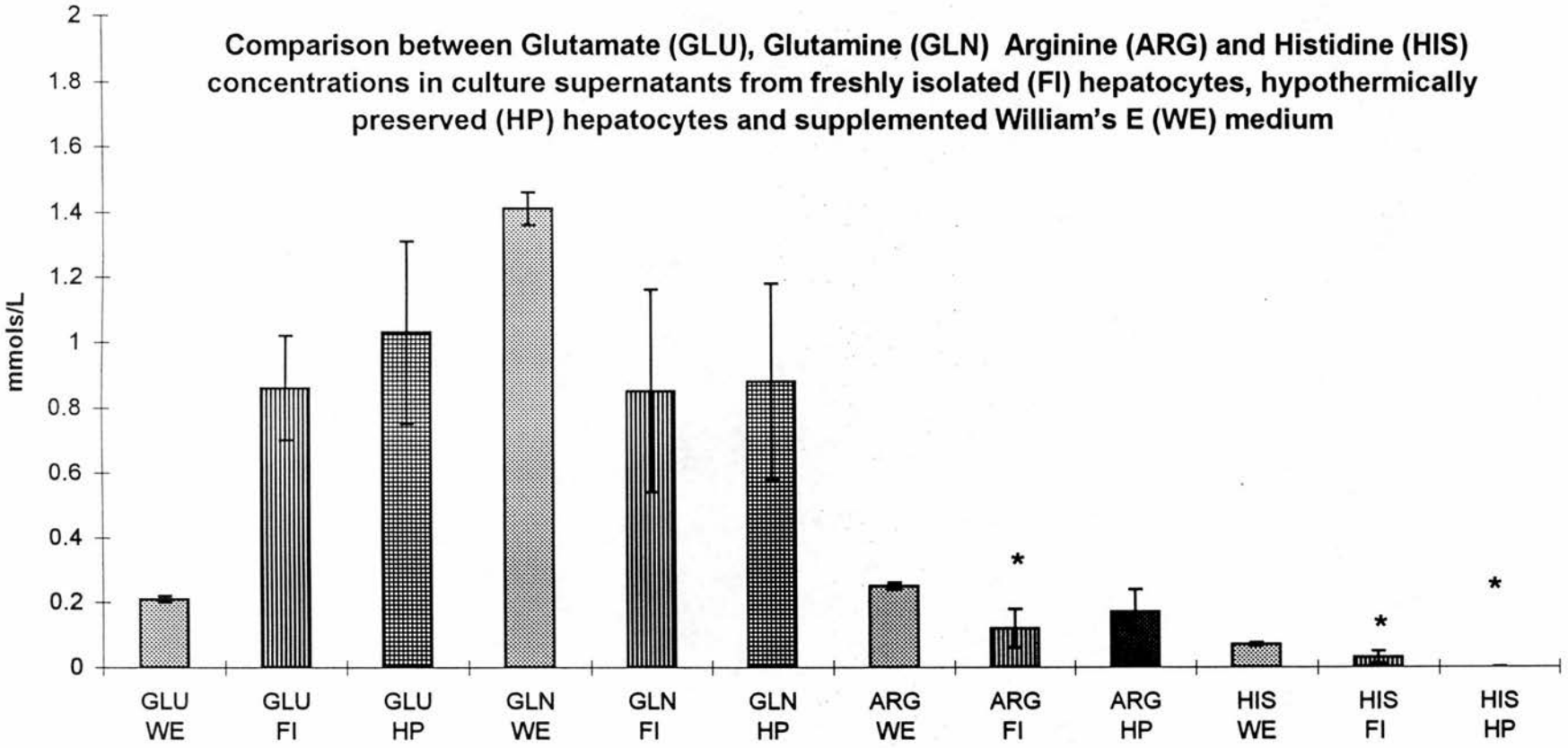
Figure 5.4 shows the levels of leucine, isoleucine, tyrosine and phenylalanine in supernatants of FI and HP cells and supplemented William's E medium for comparison

Leucine, isoleucine, tyrosine and phenylalanine are all ketogenic amino acids for the hepatocytes. Both FI cells and HP cells utilised preferentially leucine from the medium, showing a significant reduction in concentration ($p<0.003$ for FI cells and $p<0.03$ for HP cells). Small quantities of isoleucine were also taken up by FI and HP cells.

Looking at tyrosine we observed that FI cells showed significant uptake of tyrosine ($p<0.04$). In contrast,the HP cells showed no uptake of tyrosine and indeed there was some release of tyrosine from the cells back into the medium which was not statistically significant. Phenylalanine was not significantly taken up by either FI or

HP cells although there was a trend for FI cells to uptake a little more of the amino acid.

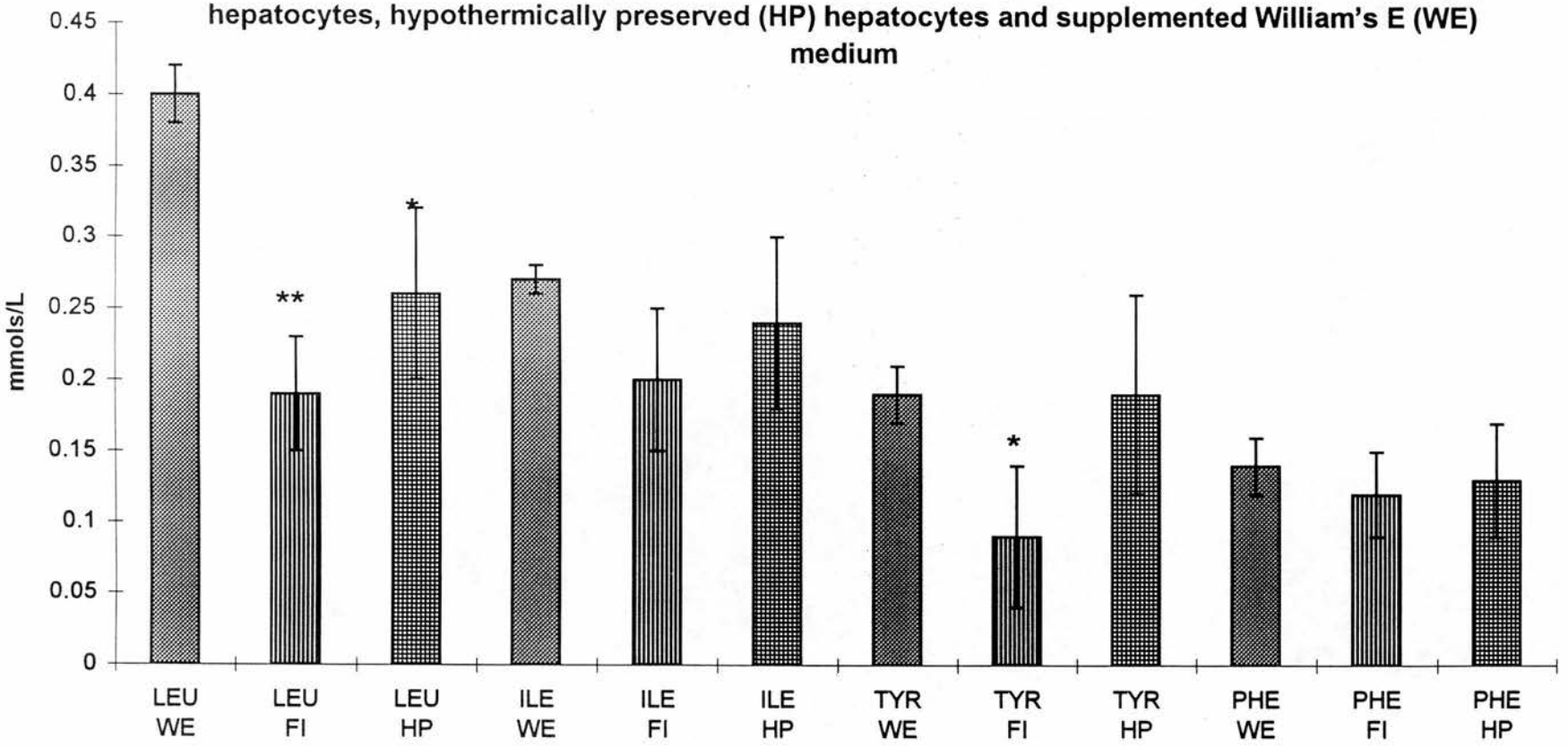
Figure 5.3



Results shown are means and error bars are SEM. Comparisons on the graph are made with previous bar. *p<0.05

Figure 5.4

Comparison between Leucine (LEU), Isoleucine (ILE), Tyrosine (TYR) and Phenylalanine (PHE) concentrations in culture supernatants from freshly isolated (FI) hepatocytes, hypothermically preserved (HP) hepatocytes and supplemented William's E (WE) medium



Results shown are means and error bars are SEM.
Comparisons on the graph are made with previous bar.
*p<0.05 **p<0.01

5.3.5 Acetate

Figure 5.5 shows the levels of acetate in supernatants of FI and HP cells. Acetate production was present both in FI and HP cells. There was significant production of acetate by HP cells but the levels of acetate production in FI cells weresignificantly higher ($p<0.001$). This may overall represent some impairment of ketogenesis in hypothermically preserved cells.

5.3.6 Succinate precursors.

Table 5.6 shows the levels of methionine and valine in supernatants of FI cells, HP cells and supplemented William's E medium for comparison. Those two amino acids are glucogenic for the hepatocytes and are used by the hepatocytes to form succinate which in turn will feed the Krebs cycle and aerobic glycolysis.

FI cells consumed significant quantities of methionine ($p<0.001$) and on average all the methionine present in the medium was taken up by the hepatocytes. HP cells showed no evidence of methionine consumption.

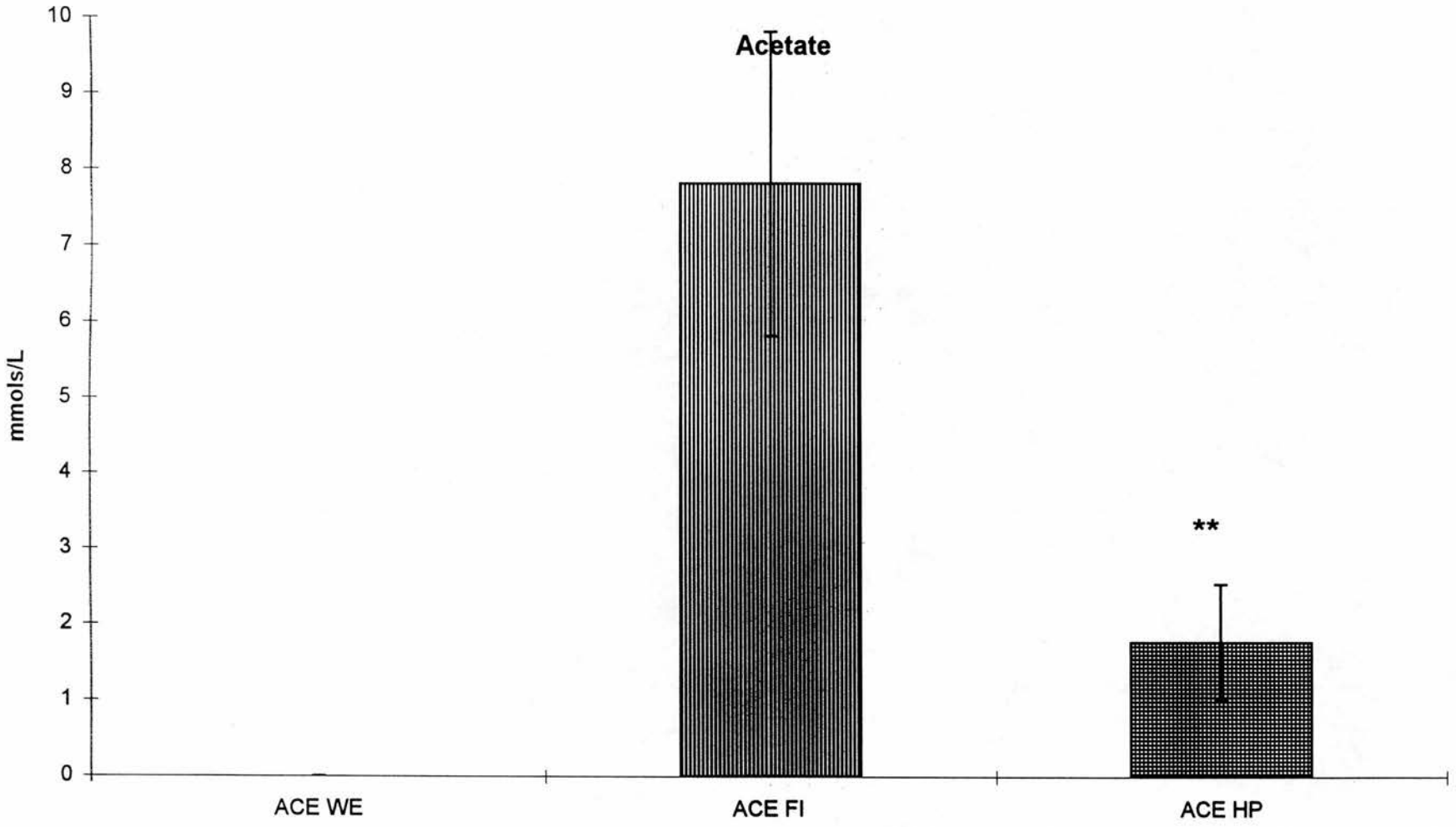
There was a small consumption of valine present in both FI and HP cells. There was a trend for FI cells to consume more valine but the resulting differences were not significant.

5.3.6 Ethanol.

Figure 5.7 shows the levels of ethanol in supernatants of FI cells and HP cells. Supplemented William's E medium does not contain any ethanol, but ethanol was readily identifiable in NMR spectra from all culture supernatants from either FI cell cultures or HP cell cultures. The ethanol concentration was elevated in all examined

supernatants. A trend of higher ethanol concentrations in the supernatants of FI cells than in supernatants of HP cells was noted but the results were not statistically significant.

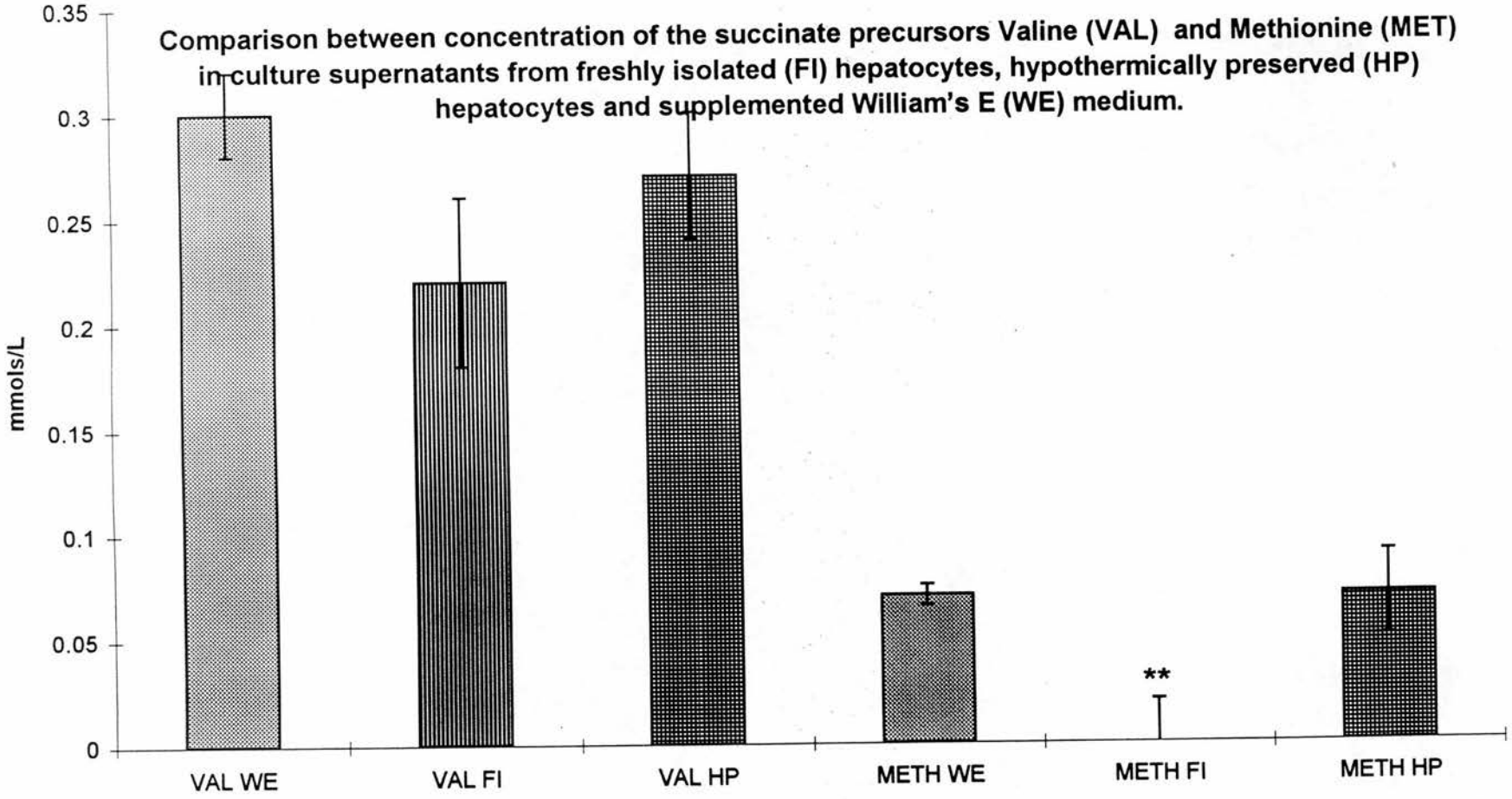
Figure 5.5



Comparison between Acetate (ACE) concentrations in culture supernatants f rom freshly isolated (FI) hepatocytes and hypothermically preserved (HP) hepatocytes

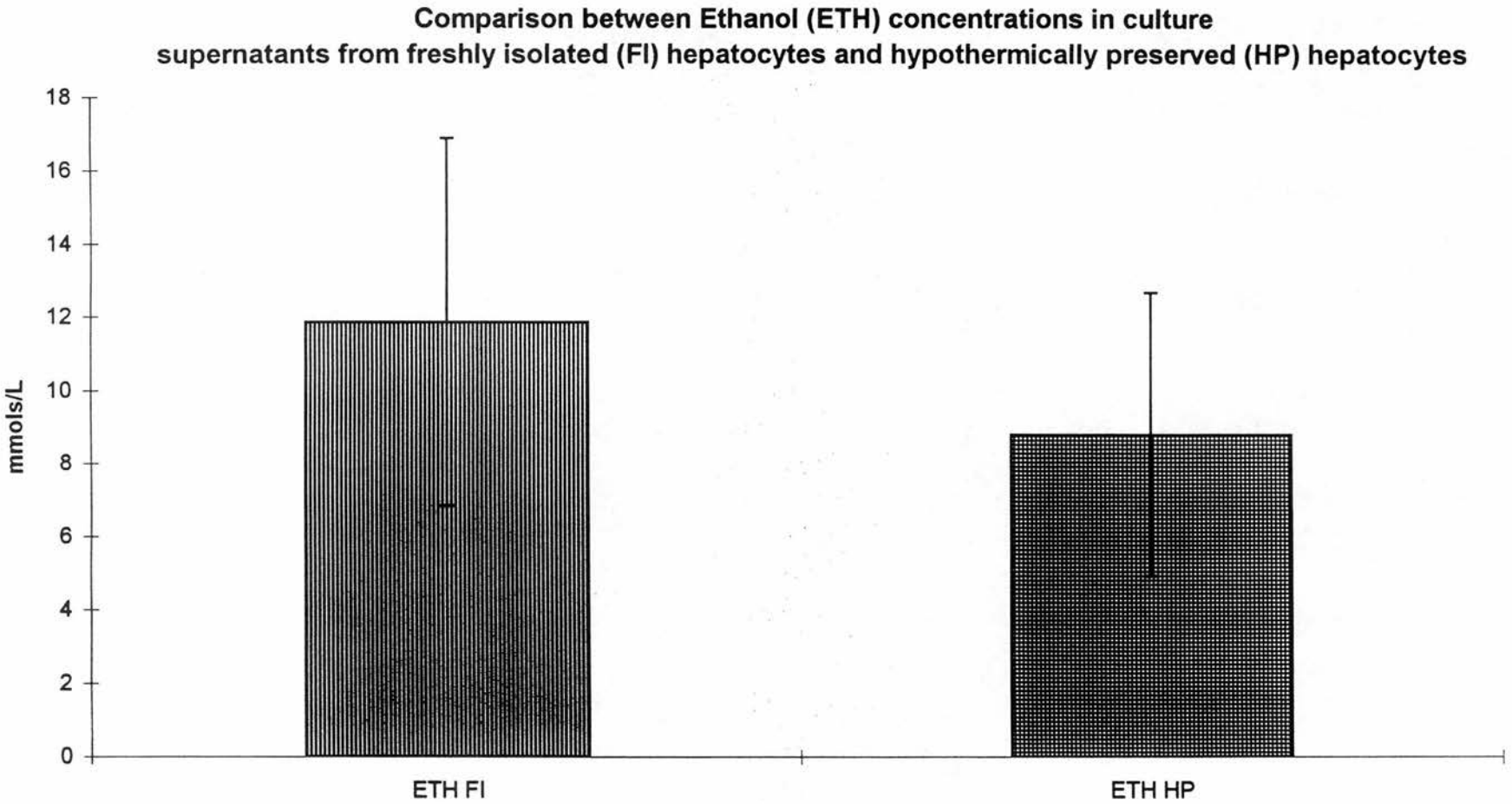
Results shown are means and error bars are SEM.
Comparisons on the graph are made with previuos bar.
**p<0.01

Figure 5.6



Results shown are means and error bars are SEM. Comparisons on the graph are made with previous bar. **p<0.01

Figure 5.7



Results shown are means and error bars are SEM.

5.4 DISCUSSION

This study was undertaken to systematically assess primary porcine hepatocyte metabolism following hypothermic preservation using ^{1}H NMR Spectroscopy. These cells were kept in moderate hypothermia at 4 $^{\circ}$ C for 48 h, before being plated. The culture supernatants were examined at 48 h after plating.

Viability was reduced from 78.1 % at isolation to 58.9% after preservation in hypothermia for 48 h. plating efficiency was also greatly reduced from 87% at isolation to 33.6% after hypothermic preservation. This represents the effect of hypothermia induced cell - injury and is in accordance with previous studies on primary porcine hepatocytes and hepatocytes of other species (Marsh et al, 1991; Poullain et al, 1992; Vara et al, 1995; Naik et al,1997).

As the plating efficiency between FI and HP cells varied greatly, it is obvious that the cell densities on the culture dishes were different. However, phase contrast microscopy studies did not show differences between the two cell populations. In addition studies in our laboratory and others comparing metabolic functions of fresh hepatocytes, over a period of 6 days confirmed that urea and albumin synthesis and galactose elimination by the cells were not significantly different despite different levels of cell densities. We thus believe that cell density would not influence the metabolic functions of hepatocytes.

We have used the Leibovitz L-15 medium as our cryopreservation medium as literature evidence suggests and our preliminary experiments confirmed that it is superior to specific hepatocyte culture and preservation media (Marsh et al, 1991, Poullain et al, 1992).

In this study, using NMR Spectroscopy, we have been able to show that major pathways of carbohydrate and aminoacid metabolism that operate in normal cells are conserved in hypothermically preserved cells.

Glucose from the glucose rich William's E medium was consumed by HP cells. The glycolytic was activated and pyruvate was produced. Although William's E contains pyruvate which FI cells utilised, it appears that HP cells are able to utilise their glucose more efficiently with net production of pyruvate and a very small production of lactate. This indicates a predominantly aerobic pattern of glucose oxidation by HP cells. A net production of alanine and a net consumption of threonine from the HP cells was on the other hand, suggestive of inactive gluconeogenesis. In contrast, FI cells utilised their alanine to feed their gluconeogenetic pathway quickly and they seemed to be unable to efficiently convert threonine to alanine. Pyruvate is the a-ketoacid that is derived from alanine. Liver cells possess the enzymatic machinery to interconvert pyruvate and alanine. It has been shown that if an excess of pyruvate is present intracellularly it can either be converted to oxaloacetate and enter the Kreb's cycle for efficient energy storage through production of high energy phosphate bonds, or be converted to lactate for fast energy release, or be converted to alanine for incorporation into protein synthesis pathways or be used for gluconeogenesis (Denton et al, 1978; Petersen et al, 1994). The observed threonine consumption was a sign of threonine conversion to alanine which is a sign of active gluconeogenesis as alanine can be converted to pyruvate and further to glucose. Although organic acids from the Kreb's cycle were not present in supernatants of the cell cultures, future studies designed to examine cell extracts from

cell cultures may be able to elucidate the question of increase turnover of Kreb's cycle.

Successful turnaround of nitrogen compounds is another key function of the hepatocytes. This was reflected in transamination reactions and urea production through the urea cycle. HP cells in culture, took up glutamine from the glutamine rich William's E medium and released glutamate into the culture supernatant. Glutamate and glutamine both play a major role in the transamination reactions involving amino acids. It has been shown that the observed pattern in the case of HP cells supernatants reflects transamination activity (Gerlach et al,1996). Uptake of histidine from the medium by HP cells shows that the cells utilise histidine to form glutamate. Uptake of arginine from the media also shows that arginine is introduced into the urea cycle. Our results confirmed that the urea cycle was active and transamination reactions occurred in HP hepatocytes. Glutamine uptake also confirmed that cells were active in DNA synthesis and repair.

Acetate production from fatty acid oxidation is characteristic of a well functioning hepatocyte (Seglen et al.1974). Although there was evidence that leucine was taken up by the HP hepatocytes the other acetyl-CoA precursors studied tyrosine, phenylalanine and isoleucine showed either minimal uptake or even some release into the medium (tyrosine). There was some production of acetate from HP hepatocytes indicating that fatty acid oxidation and ketogenesis took place but compared with FI cells the concentration of acetate after 48 h was four fold lower. It seems that all the ketogenic mechanisms from amino acid breakdown in HP cells are impaired.

Valine, isoleucine and methionine are some of the glycolytic aminoacids that the hepatocytes breakdown to feed the Kreb's cycle through succinate formation.

The fact that all amino acids showed evidence of minimal uptake is an indication that the Kreb's cycle is well fed from the hexose phosphate pathway and does not need amino acid break down for satisfactory turn over.

High levels of ethanol were observed in all culture supernatants. The possible explanations for this were discussed in Chapter 4.

Overall our results are in agreement with previous reports that HP hepatocytes maintain their differentiated functions and can be successfully revived after hypothermia (Marsh et al, 1991; Poullain et al, 1992; Vara et al, 1995; Sakai et al,1996). These results will enable researchers to use hypothermia as a temporary storage procedure for porcine hepatocytes. This could apply to the preservation of hepatocytes from other species like dogs, mice and rats. As porcine hepatocytes are more difficult to preserve and culture than hepatocytes of other species we can assume that this technique could be applied to hepatocytes of other species. Cryopreservation experiments have confirmed our hypothesis (Chesne et al, 1993, Darr et al, 1997). Although this is not a definitive method of preservation it allows scientists to maintain these cells in suspension for potential applications such as BALSS or hepatocyte transplantation. We have been unable to maintain HP cells for more that 48 h. It was clear that after 72 h viability was greatly reduced. Further studies on long term culture of HP hepatocytes are required perhaps by altering the hypothermia medium or the culture medium. Also further studies are needed on other techniques for hepatocyte storage long term such as preservation of hepatocytes in three dimensional structures in microgravity environment.

CHAPTER 6

STUDIES ON HEPATOCYTES CULTURED IN MICROGRAVITY

6.1 INTRODUCTION

During the last few decades our ability to culture isolated cells *in vitro* has been the basis for fundamental advances in cell biology. In spite of the amount of information gained in the traditional cell culture settings, it is believed that conventional tissue culture in two dimensions may be inadequate to model the complex cellular interactions that promote tissue specific differentiation. Cells maintained as homotypic population in 2-D culture quickly lose their differentiated functions (Kloth et al, 1995). This can be maintained by growing cells under the necessary environmental conditions for organ like assembly, such as cell- cell interactions in three dimensions. Three dimensional cell aggregates can be obtained in suspension cultures but the high levels of fluid shear stress encountered in conventional stirred fermentors limit the level of tissue specific differentiation of the aggregated cells (Lelkes et al, 1998).

Experiments in space flight induced microgravity can overcome the high shear forces. Indeed cells cultured in suspension under those conditions, have shown significant changes in their functions. These include increased growth rate and antibiotic resistance in bacteria, increased substrate attachment in human kidney cells and increased hormone secretion by cultured lymphocytes and macrophages and many others. Even in the absence of any direct evidence for any specific mechanisms, it has

become increasingly clear that space flight induced microgravity has profound effects on cells *in vitro* (Morrison et al, 1992).

Recently the *in vitro* generation of simulated microgravity has become possible with the High Aspect Ratio Vessels (HARV). HARVs offer a revolutionary approach to study cell functions. HARVs were developed at the National Aeronautics and Space Administration (NASA) - Johnson Space Center. HARVs allow for the cultivation of cell in an environment of low shear stress, low turbulence and no gas - fluid interface (Akins et al, 1997; Baker et al, 1997; Long et al, 1999). The principle of rotating the culture vessel allows cells to be maintained in perpetual suspension with the net gravity vector approaching zero all the time.

The key to the utility of the HARV system is that the culture vessel and the fluid contained therein approximate a solid body during rotation (Goodwin et al, 1997). When cells are added to the HARV they sink to the bottom of the vessel at a constant rate related to the gravitational field, the difference in density between the particles and the medium, the size of the particles and other factors. Rotation of the HARV results in a path for the cells determined by the combination of sedimentation and movement along with the medium. By increasing the rotation speed we can prevent the cells from reaching the bottom of the vessel. The cells appear to describe an elliptical path in the medium relative to the observer. Further increases in the rotation rate results in the diminution of the elliptical path until the cells become essentially motionless relative to the medium. At this point the vessel, contents and medium approximate a solid body. The cells maintain 3D orientations relative to each other and the surrounding medium in an apparently low shear environment. These

conditions are similar to those expected to prevail in true microgravity (Goodwin et al, 1993).

Bioartificial liver support systems can benefit from hepatocytes cultured and preserved in a suspension environment. The microgravity environment presents an exciting new modality for continuous cultures of hepatocytes in suspension. They can be easily transferred out of the rotating vessels and loaded onto the bioreactors for BALSS treatments on demand.

The aim of this study was to characterise key metabolic functions of PPHs cultured in a HARV and to assess cell aggregation and maintenance of proliferation of hepatocytes on 3D structures for the predetermined time period of 21 days.

6.2 MATERIALS AND METHODS

Hepatocyte isolation was performed as previously described

6.2.1 Hepatocyte Culture in Simulated Microgravity

Cells were seeded in the 55 ml-capacity HARV (Cellon-Sarl, Luxembourg) (Figure 6.1) at a density of $2x10^6$ viable cells / ml William's medium E at 37^0C. The medium consisted of serum-free, chemically-defined medium supplemented with 50 Glutamine; 50 mg/ml Gentomycin 50 mg/ml Penicillin and 10mg/ml Streptomycin. ng/ml L-EGF; 10μg/ml porcine insulin; 1 μmol/L dexamethasone; 2 mmol/L L- The HARV was connected to the horizontally rotating axle of the RCCS which pumped incubator gas via a central aperture, across a semi-permeable gas exchange membrane within the HARV. The RCCS-HARV assembly was placed in a humidified incubator under a 95% air: 5% CO_2 atmosphere. Next day (day 1), the medium from the

HARV was aspirated and fresh medium added, which was subsequently changed every 3 days. The optimal rotational speed of the HARV was determined by adjusting the RCCS rotational speed such that the aggregates maintained a free-fall, quasi-stationary orbit within the culture vessel. The rotational speed varied according to both aggregate size and the corresponding sedimentation coefficient, however by day 6 of culture, rotational speed was set between 10-15 rpm.

For changes of supplemented William's E medium a 0.5 cm fill port was used using a suction aspirator. When changing the medium, the cells were covered by a small amount of medium to minimise cell damage. Media samples were taken daily via a syringe attached to tone of the luer-lock syringe ports and stored at -70^{0}C until NMR analysis.

Morphology was assessed using a Zeiss inverted microscope (Zeiss, Germany) under phase-contrast on both day 0 and day 21 of culture. Cell purity was determined by cytospin (x200g, 5 mins) of $5x10^5$ freshly isolated cells and staining with haemotoxylin + eosin / Periodic Acid Schiff (PAS), followed by counting cells in 3 separate fields for 3 separate isolations.

6.2.2 NMR spectroscopy.

NMR Spectroscopy monitoring was performed as discussed in Chapter 2. Sample preparation, data acquisition and quantitation of compound concentrations were performed as discussed in Chapter 2.

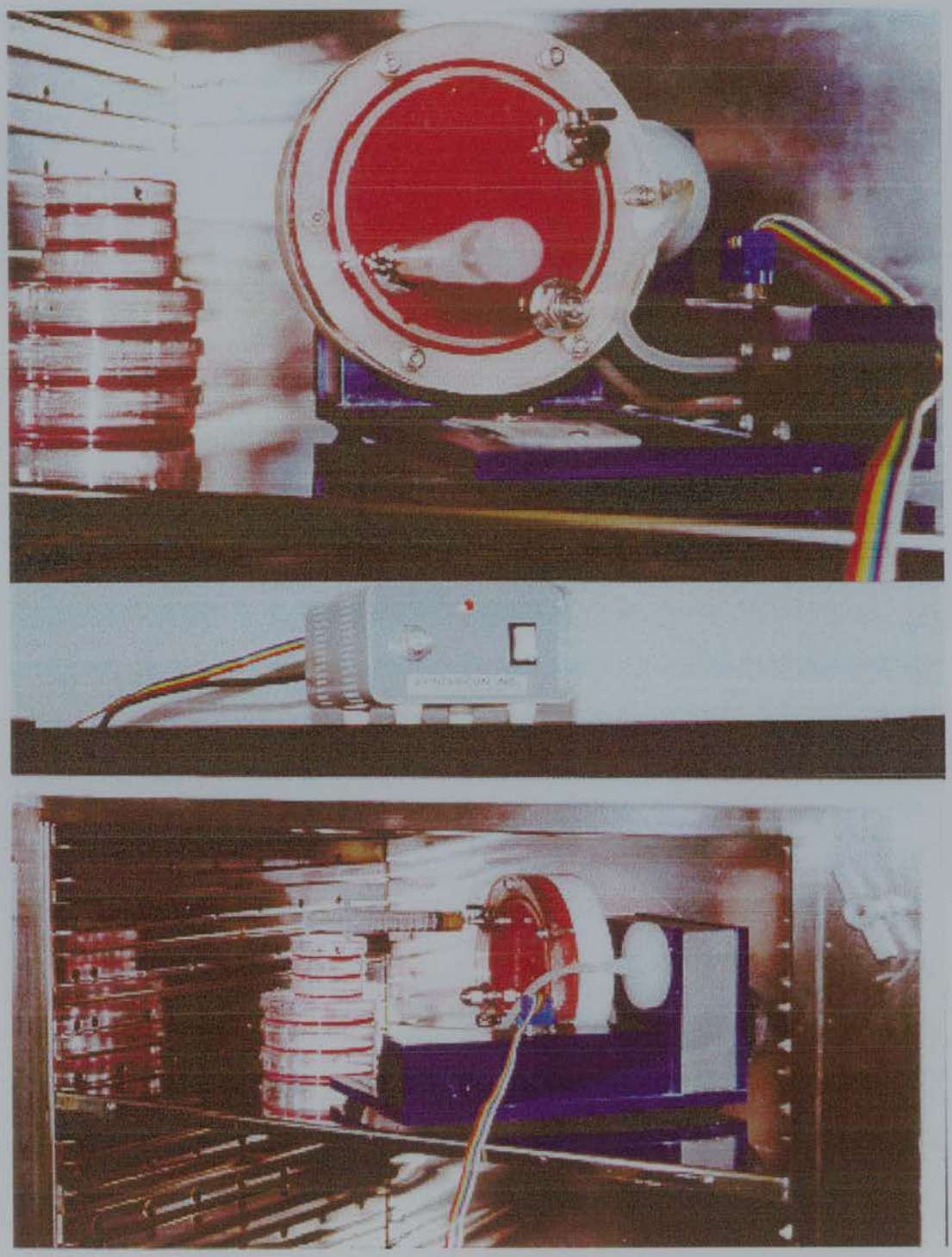


Figure 6.1 The High Aspect Ratio Vessel mounted on the Rotary Cell Culture System

6.2.3 Statistical analysis

Comparisons between freshly isolated hepatocytes maintained in the HARV environment from the same isolation were made at different time points of the experiments. Six consecutive cultures were used as the basis of our experiments. The concentration results obtained represent the mean of those six experiments. To compare between groups we used the Tukey test based on the one factor independent measures ANOVA. The Bonferoni correction was used if more than two groups were compared. A p value of < 0.05 was taken as significant (two-tail test of significance). Results are expressed as mean ± standard error of the mean

6.3 RESULTS

Cell viability, determined by trypan blue exclusion following isolation (day 0) was 85±6%. The cell suspension was composed of single cells and small aggregates of between 5-20 cells showing bright cytoplasm, sharply defined borders, commonly with round or polygonal shape and little physical damage (blebbing) under phase contrast microscopy (Figure 6.2). Hepatocyte purity for fresh cells was 95±3% (n=3) H&E staining showed gross morphology of cell aggregates at day 13 and 21; PAS staining revealed hepatocyte glycogen content at each time point.

Figure 6.3 shows a 1H NMR sperctrum of the cutlure medium from a microgravity experiment as cells are in culture for 3 days

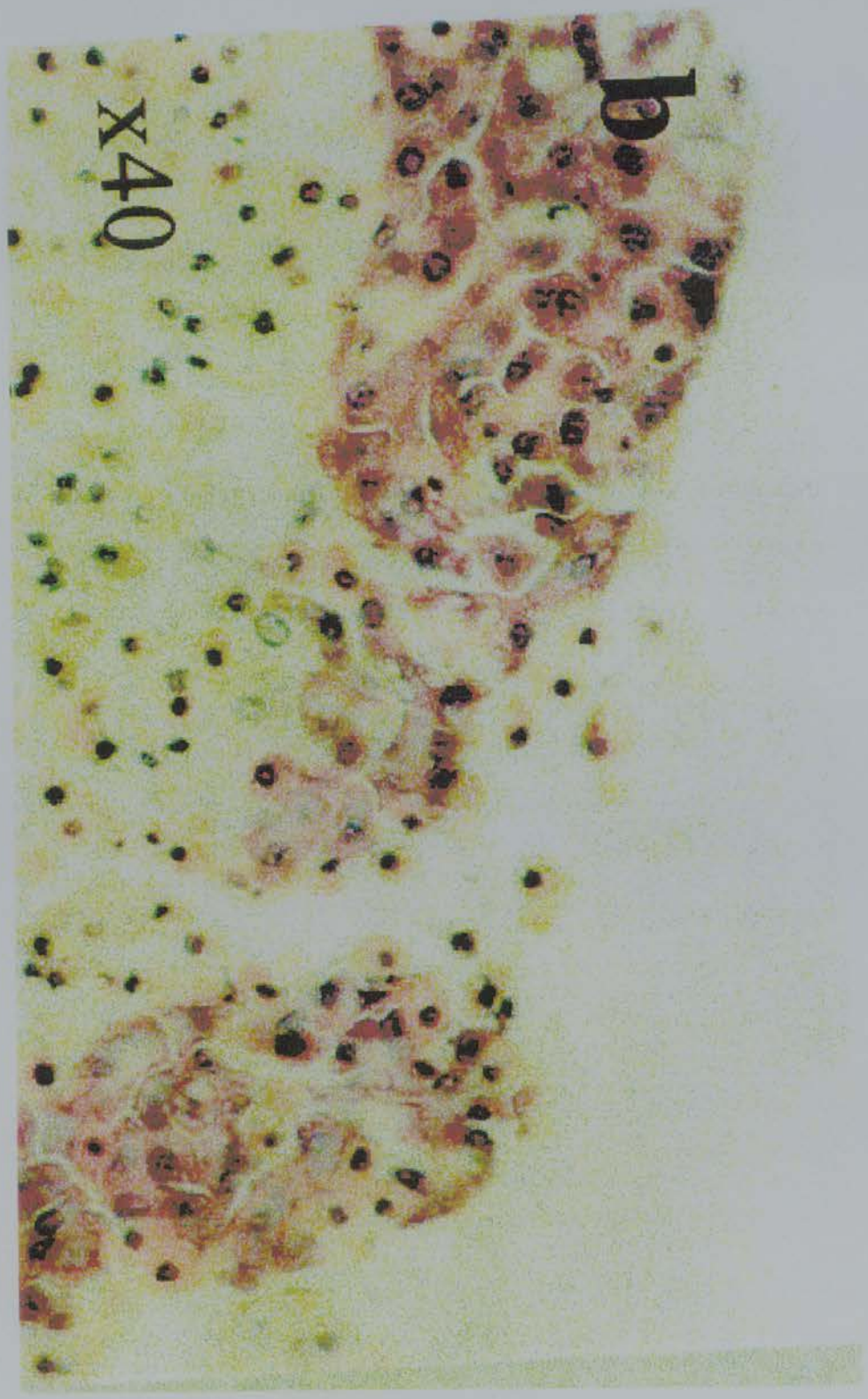
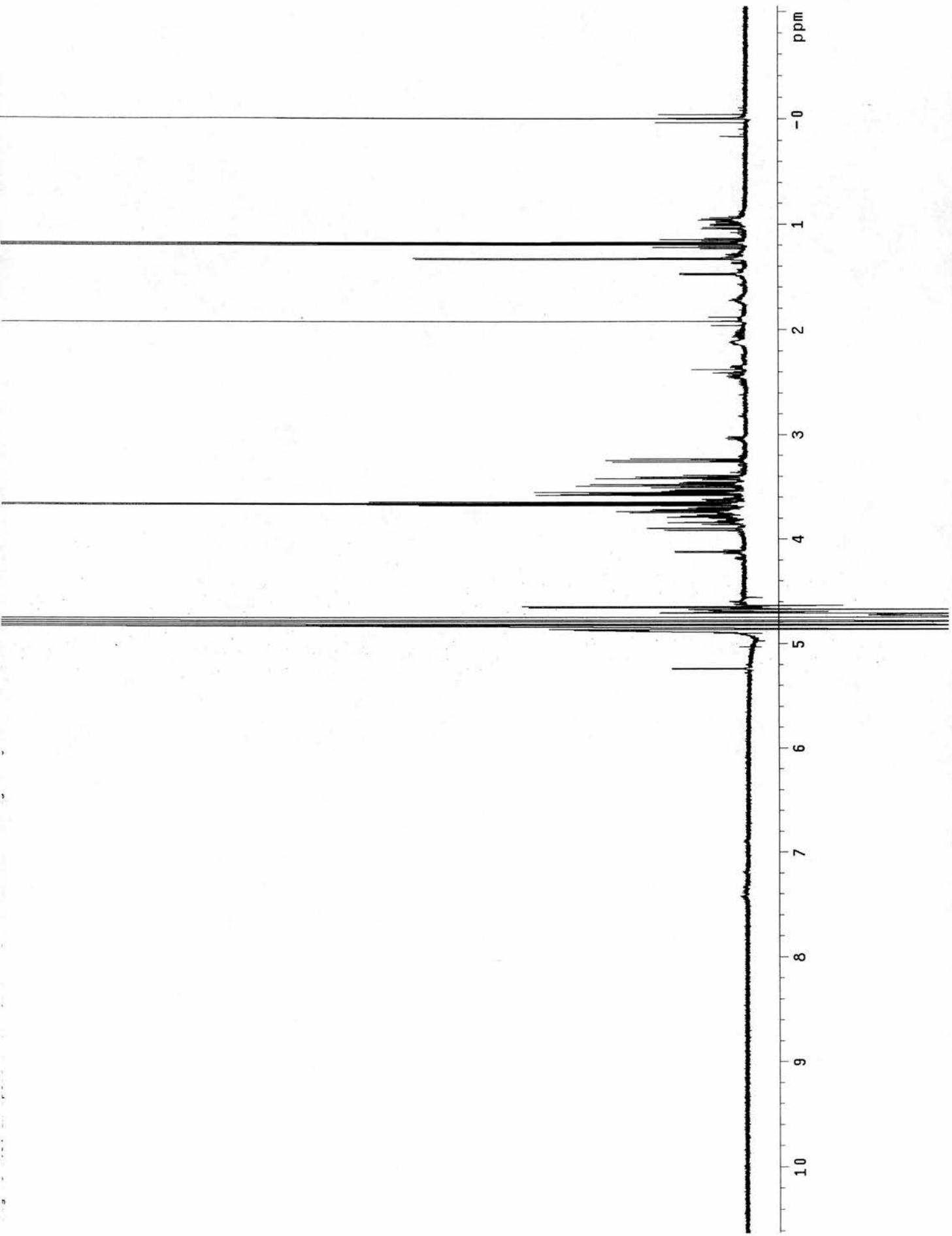


Figure 6.2 Three dimensional aggregate of hepatocytes in the HARV.


ppm
-0
1
2
3
4
5
6
7
8
9
10

6.3.1 GLUCOSE METABOLISM.

Figure 6.3 shows the results for glucose and lactate comparing concentrations between different days. Figure 6.4 shows the results for pyruvate and glucogenic amino acids comparing concentrations between different days

6.3.1.1 GLUCOSE.

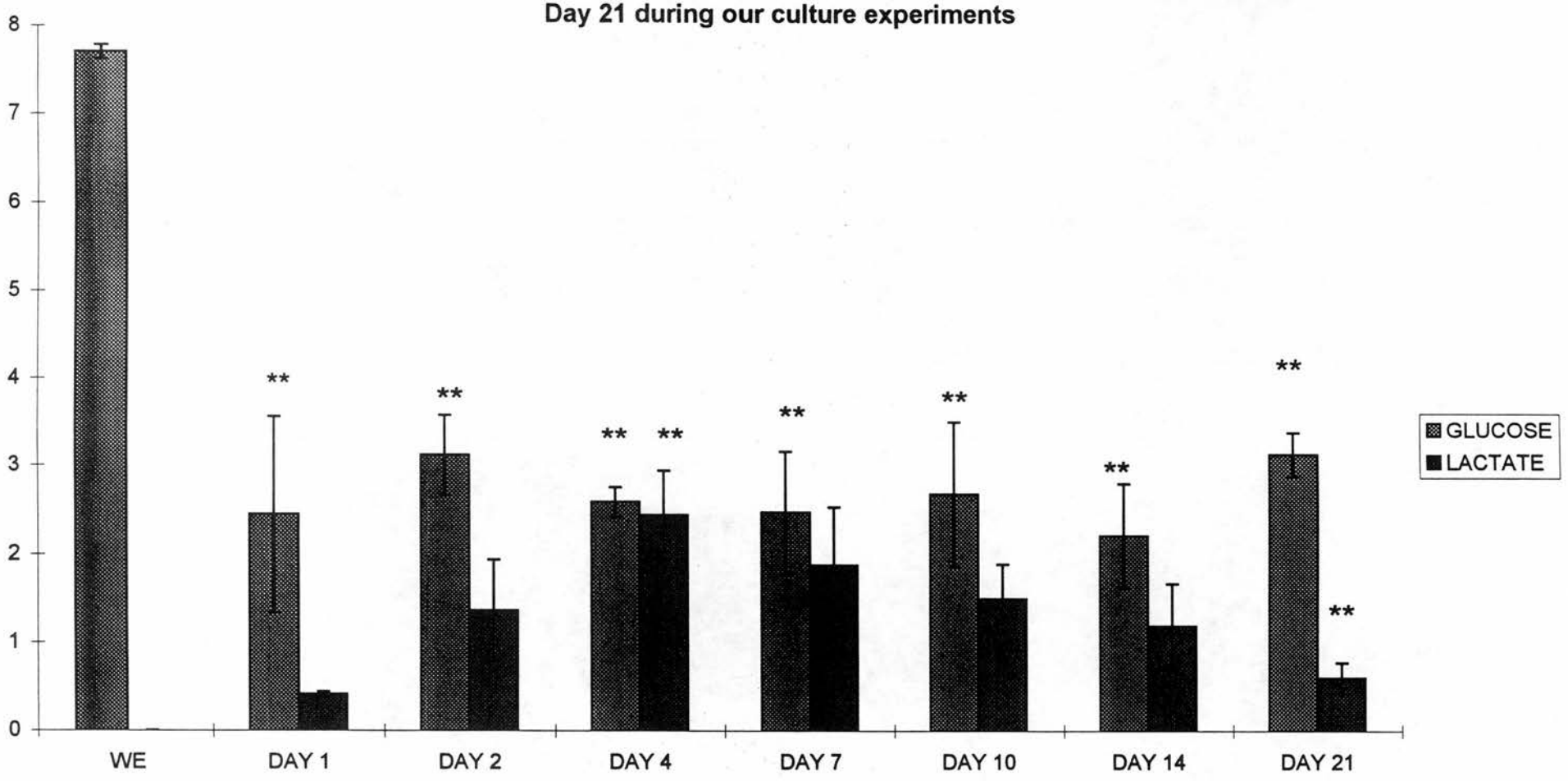
There is statistically significant consumption of glucose compared to the baseline value on all days ($p<0.01$ on all measurements). The biggest consumption is seen on day 14 and the smallest in day 21. Between days 4 and 10 glucose consumption plateaued. Overall there were no statistically significant differences between days as measured by one factor independent measures ANOVA.

6.3.1.2 LACTATE

There was lactate production at all time points sampled. There was a small production on day 1 which then increased to reach a peak on day 4 and then steadily decreased up to day 21, when lactate concentration was similar to the one on day 1. There were statistically significant differences in concentrations between day 4 and day 1 ($p<0.0029$). between day 4 and day 21 ($p<0.017$) and day 7 and day 1 ($p<0.033$).

Figure 6.4

Comparison between concentrations of Glucose and Lactate, in the culture medium from Day1 to Day 21 during our culture experiments



Results shown represent means and error bars represent SEM.
Comparisons are made with previous day's results. *p<0.05 **p<0.01

6.3.1.3 PYRUVATE

Pyruvate was consumed on all days apart from day 14 when there was net pyruvate production from the hepatocytes cultured. The biggest consumption is manifested in day 1. Pyruvate concentration in the supernatant was also low on day 10. Between days 2 and 10 pyruvate concentration plateaued. The ANOVA test shows statistical significance between concentrations on different days ($F<0.03$). The Tuckey test confirmed that there were statistically significant differences between day 1 and day 14 ($p<0.03$) and day 10 and day 14 ($p<0.02$).

6.3.1.4 ALANINE

There was consumption of alanine on days 1, 2, 4 and 10 and alanine production on days 7, 14 and 21. The lowest alanine concentration was exhibited on day 2. The maximum alanine concentration was reached on day 21. There were statistically significant differences in alanine concentration between day 2 and day 14 ($p<0.009$) and between day 2 and day 21 ($p<0.029$)

6.3.1.5 THREONINE

There was consumption of threonine at all time points sampled. There was a big variation of the amount of threonine that was consumed. If we look at the one way ANOVA test between days the results were highly significant ($F<0.00003$). Table 6.1 shows the statistically significant differences between concentrations on different days using the Tukey test. There was a very small consumption of threonine on the first day, on day 14 and on day 21. Consumption peaked on day 4 and steadily decreased

thereafter. The pattern of concentrations was opposite to and fitted with that for lactate.

TABLE 6.1

Statistically significant differences between concentrations of threonine on different days of the experiments using the Tukey test.

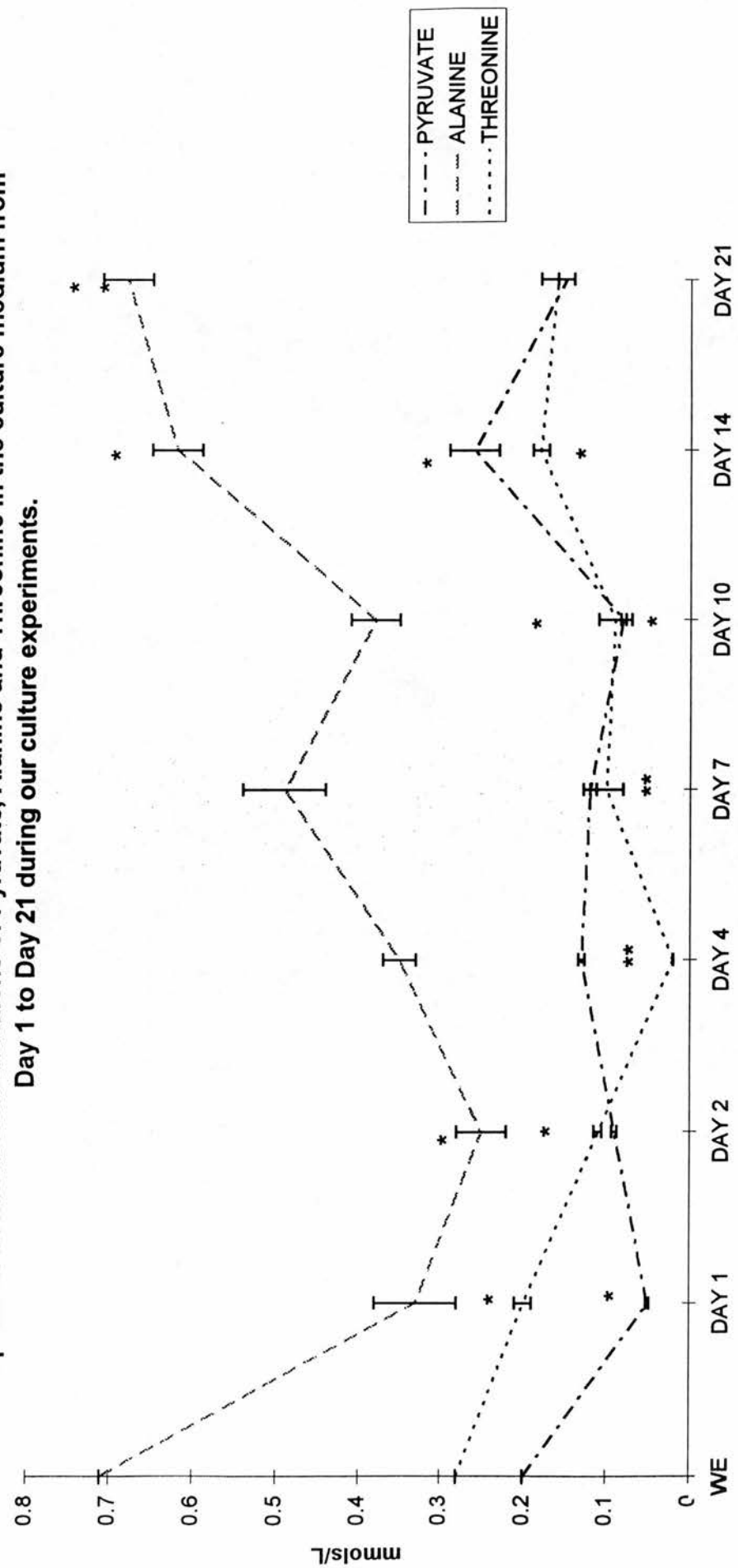
THREONINE

ANOVA SIGNIFICANCE 0.000023

DAY 1 vs DAY 2	$p<0.022$
DAY 1 vs DAY 7	$p<0.00053$
DAY 1 vs DAY 10	$p<0.002$
DAY 2 vs DAY 14	$p<0.022$
DAY 4 vs DAY 1	$p<0.00000012$
DAY 4 vs DAY 2	$p<0.011$
DAY 4 vs DAY 7	$p<0.0037$
DAY 4 vs DAY 10	$p<0.0015$
DAY 4 vs DAY 14	$p<0.0000028$
DAY 4 vs DAY 21	$p<0.000039$
DAY 7 vs DAY 14	$p<0.0033$
DAY 10 vs DAY 14	$p<0.002$

Figure 6.5

Comparison between concentrations of Pyruvate, Alanine and Threonine in the culture medium from Day 1 to Day 21 during our culture experiments.



Results shown represent means and error bars represent SEM. Comparisons are made with previous day's results. *p<0.05 **p<0.01

6.3.2 GLUTAMINE SYNTHESIS

Figure 6.5 shows the overall results for histidine and arginine which are two glutamate precursors in the cell, glutamate and glutamine which is the end product of this chain and is utilised by the cells in DNA synthesis

6.3.2.1 HISTIDINE.

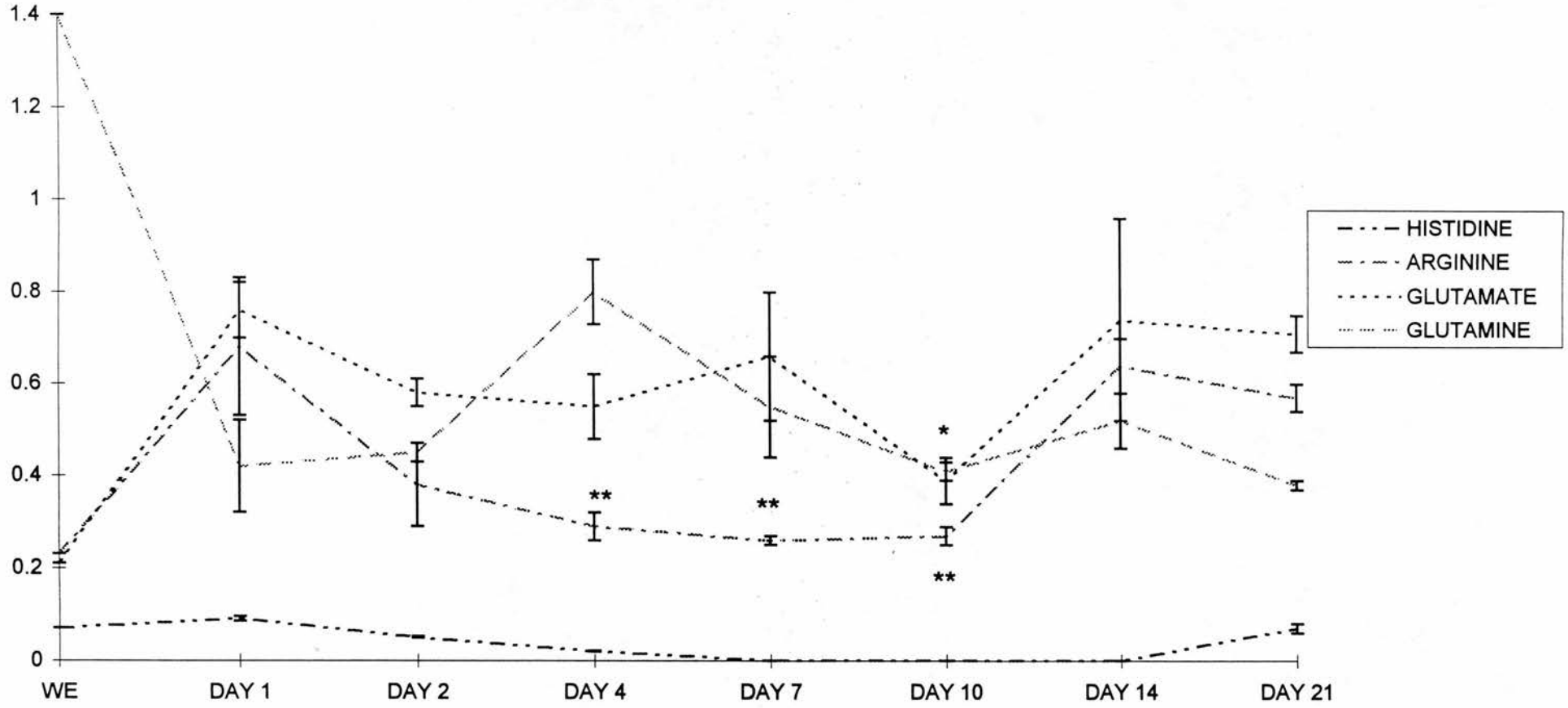
There was consumption of histidine on all days sampled except for day 1 and day 21, when consumption was minimal or non-existent, but there was no evidence of production of histidine. Consumption increased from day 1 reaching a peak on day 7. Between days 7 and 14 there was consumption of all the histidine which is present in the medium. One way ANOVA showed that those differences were not statistically significant.

6.3.2.2 ARGININE

Contrary to the consumption observed with histidine there was production of arginine at most of the time points sampled apart from days 7 and 10 when there was minimal consumption. Day 1 showed high production of arginine as was the case on days 14 and 21, with a peak on concentration on day 14. There were statistically significant differences between concentrations between day 14 and day 4 ($p<0.002$)., between day 14 and day 7 ($p<0.0000$), between day 14 and day 10($p<0.0007$), between day 21 and day 7 ($p<0.02$) and between day 1 and day 7 ($p<0.037$).

Figure 6.6

Comparison between concentrations of Histidine, Arginine, Glutamate and Glutamine in the culture medium from Day 1 to Day 21 during our culture experiments



Results shown represent means and error bars represent SEM. Comparisons are made with previous day's results. *p<0.05 **p<0.01

6.3.2.3 GLUTAMATE.

Glutamate concentration overall exhibited the same patterns as arginine concentrations. There was production of glutamate at all time points sampled. Production of glutamate was maximal on day 1 and was comparable to this on days 14 and 21. Between days 2 and 10 there was a small production of glutamate exhibited and the amounts produced were similar. There was one statistically significant differences between days between day 1 and day 10 ($p<0.031$).

6.2.3.4 GLUTAMINE.

There was consumption of glutamine at all time points sampled. The pattern of concentrations was inverse to that of glutamate. There was a big consumption manifested on day 1 which decreased to a minimum on day 4. Between day 4 and day 10 it plateaued at the same level. It then increased to reach a maximum peak on day 21. Overall One way ANOVA showed that those differences were not statistically significant.

6.3.3 KETOGENESIS

Figure 6.7 shows the overall results for phenylalanine, tyrosine, leucine and isoleucine which together with tryptophan and lysine are the acetyl-CoA precursors. Figure 6.8 shows the overall production of acetate by the cultured cells during the experiments.

6.3.3.1 PHENYLALANINE.

There was production of phenylalanine by cells as a result of protein breakdown on all days sampled apart from day 1 when there was minimal consumption of phenylalanine. The rates of phenylalanine production varied between days. They showed an increase in concentration from day 1 to day 4 and then plateaued, with a small decrease on day 21. One way ANOVA showed statistically significant differences between days ($F<0.02$). The Tukey test confirmed statistically significant differences between day 1 and day 7 ($p<0.028$), between day 1 and day 10 ($p<0.0012$) and between day 1 and day 14 ($p<0.0008$).

6.3.3.2 TYROSINE

Tyrosine concentration showed an inverse pattern to the concentrations of phenylalanine. There was consumption of tyrosine on all days sampled apart from day 1 when there was a small production. Days 10 and 14 showed the biggest uptake of tyrosine from the medium. The concentration then increased and on day 21 the uptake was minimum. There were statistically significant differences between concentrations of tyrosine between day 10 and day 1 ($p<0.0001$), between day 10 and

6.3.3.3 LEUCINE

There was significant consumption of leucine compared to the baseline concentrations of the medium at all days. This has reached statistical significance at all days ($p<0.01$ at all time points). Overall over the time scale of the experiments the concentration of leucine of the supernatant remained stable and there were no statistically significant differences between the time points sampled.

6.3.3.4 ISOLEUCINE

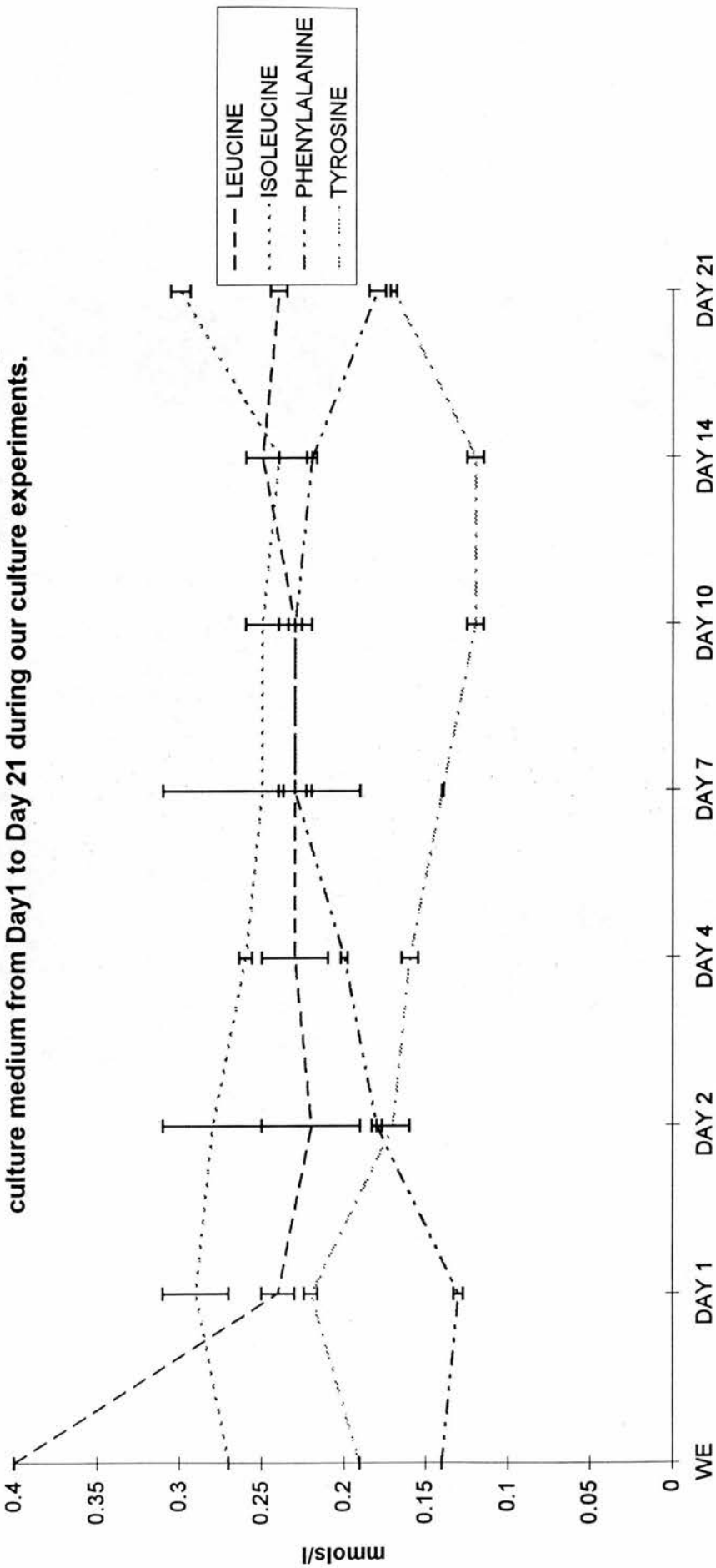
Overall there were no significant changes from baseline concentrations on isoleucine concentrations throughout the experiments. They remained relatively stable with small amounts of isoleucine taken up or released on different days. No statistically significant differences were exhibited between days as well. Cells in the RCCS did not use isoleucine for their needs.

6.3.3.5 ACETATE.

There was production of acetate at all time points sampled. Acetate production was elevated on day 1, reached its peak on day 2 and then decreased on day 4 to a plateau through to day 10. Then it reached its minimum on day 14 and on day 21 it was similar to day 14. There were statistically significant differences between acetate concentrations between day 14 and day 1 ($p<0.013$), between day 14 and day 2 ($p<0.0001$), between day 14 and day 7 ($p<0.009$) and between day 21 and day 2 ($p<0.013$).

Figure 6.7

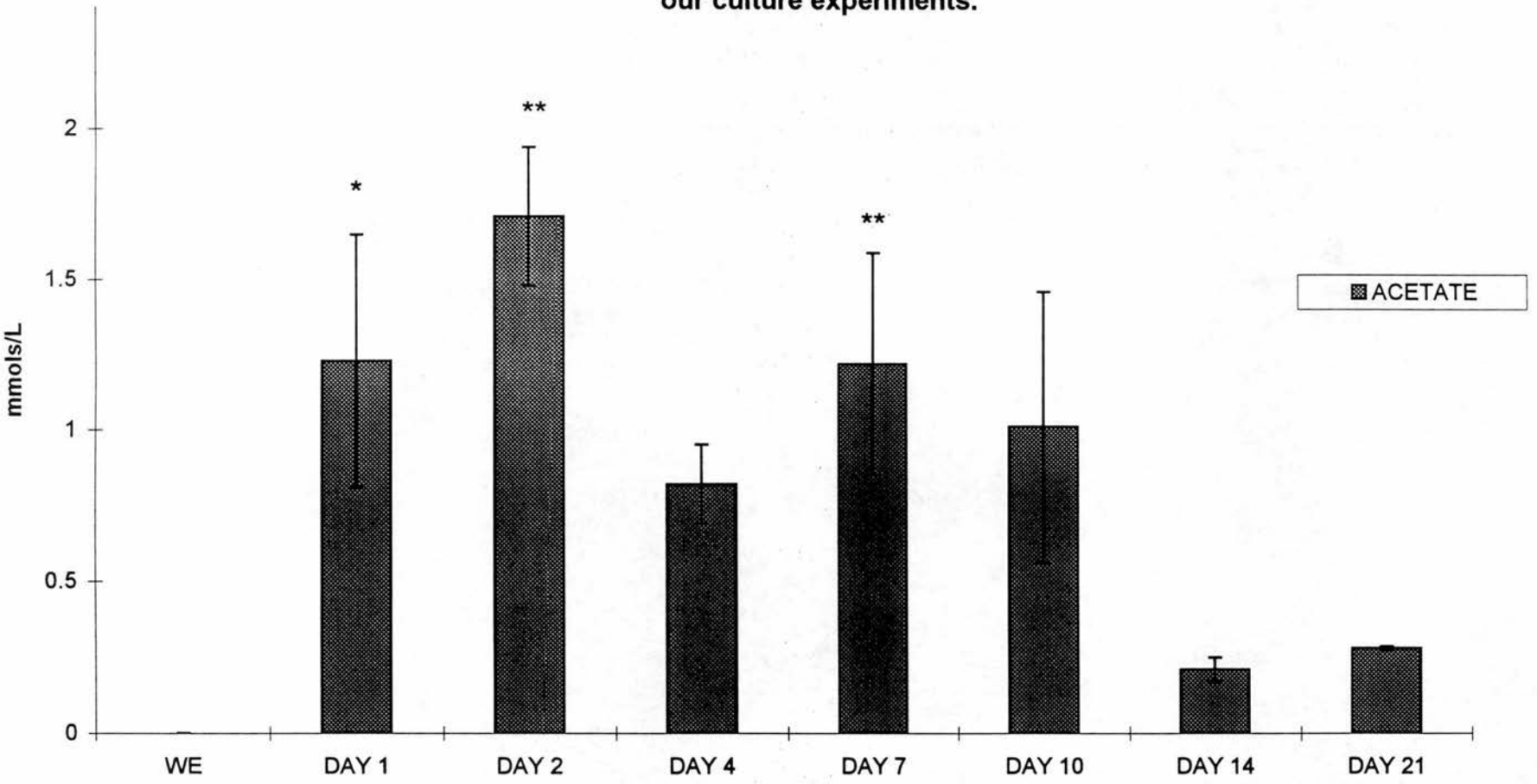
Comparison between concentrations of Leucine, Isoleucine, Phenylalanine and Tyrosine in the culture medium from Day1 to Day 21 during our culture experiments.



Results shown represent means and error bars represent SEM. Comparisons are made with previous day's results.

Figure 6.8

Comparison between concentrations of Acetate, in the culture medium from Day 1 to Day 21 during our culture experiments.



Results shown represent means and error bars represent SEM. Comparisons are made with the smallest concentration seen on day 14. *p<0.05 **p<0.01

6.3.4 SUCCINATE PRODUCTION

Succinate is an essential intermediate acid of the Kreb's cycle. Valine, isoleucine and methionine are the amino acids that are transformed to succinate by the hepatocytes to feed the Kreb's cycle. Figure 6.9 shows the concentrations of those amino acid s at different time points during our run of experiments.

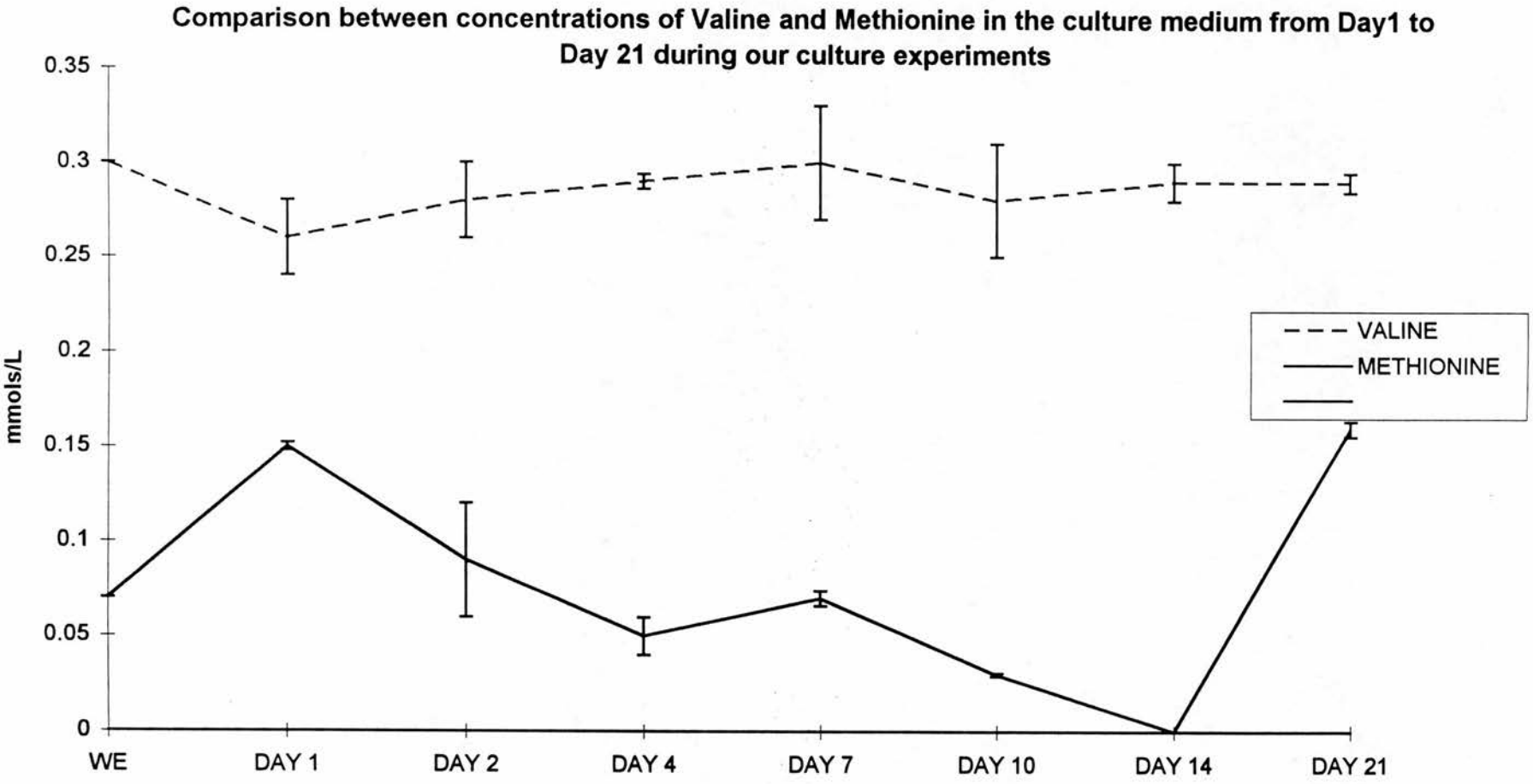
6.3.4.1 VALINE

There was a small consumption of valine present at all time points sampled. There were no statistically significant differences neither between concentrations of valine on different days nor between concentrations of valine sampled and the baseline concentration of William's E medium. The concentration of valine in the supernatants remained relatively stable with minimal amounts consumed on every sample.

6.3.4.2 METHIONINE.

The pattern exhibited by methionine was of consumption on days 1, and 21 with production of methionine from protein breakdown between days 4 and 14. The consumption peaked on day 14 when all the available methionine was consumed by the hepatocytes. One way ANOVA did not reveal statistically significant differences between concentrations on different days.

Figure 6.9

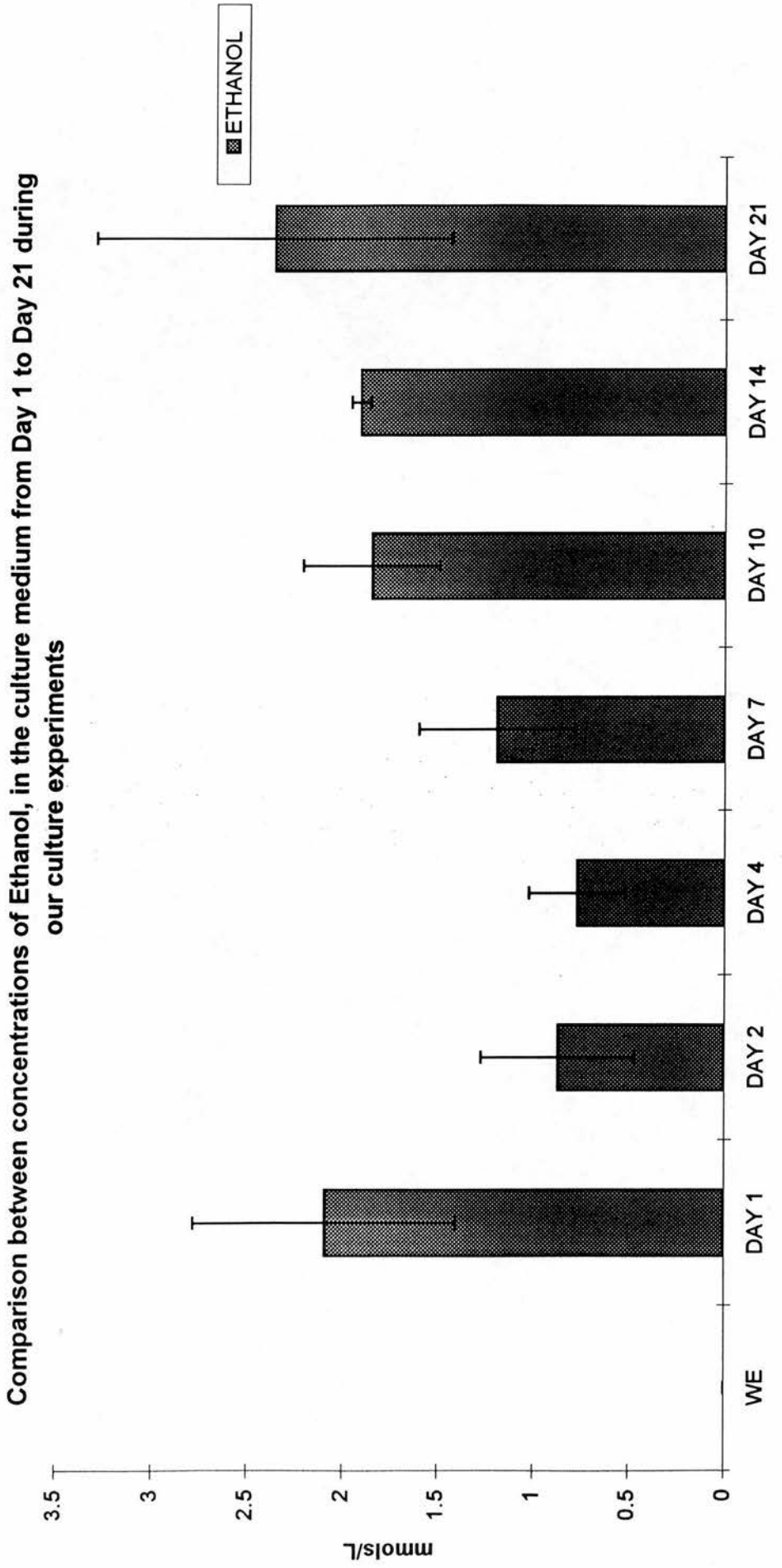


Results shown represent means and error bars represent SEM.

6.3.5 ETHANOL

There was significant ethanol production on all days at all time points sampled (Figure 6.10). The maximum concentration was exhibited on day 21. Ethanol concentration started high on day 1 then subsequently diminished to reach a minimum on day 4 and then increased to its peak on day 21. There was a statistically significant difference between concentrations between day 1 and day 4 ($p<0.02$).

Figure 6.10



Results shown represent means and error bars represent SEM.

6.4 DISCUSSION

During the last decade, with the advent of the HARV system, many different types of cells and cell lines have been successfully cultured in simulated microgravity conditions (Ingram et al, 1997; Jessup et al; 1997).

Our study examined maintenance of the differentiated metabolic functions of primary porcine hepatocytes in a simulated microgravity environment. We were able to show that cells in HARVs not only survive for long periods of time but are also able to maintain key metabolic functions.

Our findings were entirely consistent with those from a recently published paper (Khaoustov et al, 1999), where the authors showed that human liver cells cultured in simulated microgravity were viable for 60 days and aggregated to tissue like structures. They also showed that albumin and urea were produced and glucose was consumed.

Our study showed that hepatocytes not only consume glucose but they are able to feed both the Krebs and the Cori cycles. Looking at glucose metabolism in particular, there was a pattern emerging showing that during the first 7 to 10 days there was lactate production, pyruvate consumption, alanine consumption and threonine consumption. This is a pattern observed in cells breaking down glucose anaerobically for their energy requirements and active gluconeogenesis. From day 10 onwards though this pattern was reversed. There was diminishing lactate production, pyruvate production, alanine production and diminishing threonine consumption. This is a pattern observed in cells breaking down their glucose aerobically for their energy requirements and not very actively using glucogenic amino acids for gluconeogenesis .

This observation and similar others on key metabolic pathways detailed below have enabled us to put forward a hypothesis that cells grow quite happily in the RCCS. After an initial period of acclimatisation at the time point when static cell cultures drift towards cell death, cell aggregates formed in the RCCS switch from the anaerobic pattern of metabolism to the more efficient aerobic pattern. They exhibit the same pattern of metabolism until the end of the experiments.

Looking at glutamine synthesis we observed that glutamate can be produced either from the urea cycle through arginine breakdown, or directly from histidine or through transamination from α- ketoglutarate, which produces the bulk of intracellular glutamate. The results showed that glutamate production closely followed histidine consumption. This only produces a small proportion of the overall glutamate production by the cells. We suggest that transamination activity and production of glutamate overall, was proportional to glutamate production from histidine.

The hypothesis of cells acclimatising at the microgravity environment for the first 10 days and then continuing their life cycles in a friendly environment is suggested by those results. The glutamine consumption pattern being inverse to what the glutamate production pattern was, suggested that cells post day 7 were consuming glutamine for their DNA synthesis. They were as such dividing until day 21 when our experiments were terminated.

Looking at ketogenesis we observed a significantly reduced acetate production compared to acetate production from freshly isolated cells cultured in static cultures. We also observed contrary to what has been reported on static cultures phenylalanine production from protein breakdown. It is well established that

hepatocytes tend to convert phenylalanine to tyrosine which is further converted to acetyl- CoA and fumarate. The increased concentration of phenylalanine in the media and in the cells accelerates the turnover of tyrosine and its conversion to metabolites more useful to the cell. The results are entirely in keeping with this hypothesis.

Overall the pattern of ketogenesis proves again the theory of cells acclimatising in the RCCS environment. Ketone bodies are the export products of liver cells to other organs that need ketone bodies to survive. The microgravity cultures exhibited high acetate production at the beginning. As only hepatocyte aggregates existed in the system there was no organ target to export ketone bodies. Eventually, acetate production diminished and reaching its minimum on day 14 and remaining small until the end of the experiments.

There is no evidence of a big succinate production as all precursors of succinate seemed not to be taken up in big quantities. Overall it does not seem that the Kreb's cycle was fed from succinate production under our experimental conditions.

Looking at the branch chain amino acids as an entity it becomes clear that cells use preferentially leucine for their requirements. The most plausible explanation is that as all three branch chain amino acids are using the same transporter to enter the hepatocyte , leucine is preferentially taken up because its concentration in the extracellular milieu is the highest. Until the three branch chain amino acids are in concentration equilibrium in the medium leucine is almost exclusively taken up and as a net result the concentrations of the three branch chain amino acids in the extracellular supernatant are almost identical.

Overall our results show that primary porcine hepatocyte can be happily grown in microgravity conditions for up to three weeks. This may have implications for BALSS as they can provide an active biosubstrate for the systems. We have shown that this culture modality overcomes the initial disorder in cellular amino acid metabolism which is critical in long term cultures of porcine hepatocytes (Gerlach et al, 1996). Further studies are urgently required to scale up this model for growth of substantial quantities of hepatocytes and studies without time limits would need to be performed to ascertain the feasibility of long term culture and maintenance of these hepatocytes in culture.

CHAPTER 7

METABOLIC ABNORMALITIES IN PATIENTS WITH FULMINANT HEPATIC FAILURE

7.1 INTRODUCTION

Fulminant Hepatic Failure (FHF) is a clinical syndrome characterised by massive hepatocellular dysfunction, in the absence of chronic liver disease, resulting in hepatic encephalopathy. It is a fairly uncommon syndrome and has a high mortality approaching 80% in some series (Schiodt et al, 1999). The prognosis depends on aetiology and concomitant liver disease. Remarkably, the acutely failing liver has a potential to regenerate without any permanent sequelae.

The most frequent cause of fulminant hepatic failure in the UK is paracetamol overdose (POD). The clinical syndrome in patients with FHF following POD is quite distinct and differs to other aetiologies. Paracetamol is hepatotoxic but also nephrotoxic and nephrotoxicity can be a late feature. The time course of fulminant hepatic failure after POD is usually accelerated and there is evidence that the metabolic abnormalities occur relatively early. Most series with FHF patients will consider POD patients separately and they will apply different prognostic criteria to them.

Other causes include reactions to drugs, viral hepatitis, Wilson's disease, Budd - Chiari syndrome etc. Hepatitis A is the commonest cause world wide (Plevris et al, 1998).

The clinical course is that of progressive multi organ failure. There is cerebral dysfunction with hepatic encephalopathy, coagulation abnormalities, haemodynamic abnormalities, renal failure and infections superimposed to the liver failure. Unless Orthotopic Liver Transplantation (OLT) is attempted, the prognosis is grave. OLT seems to be the only effective means of therapy in those unfortunate patients whose prognosis is poor. No pharmacological or other modality seem to be as effective as OLT. In recent series, the 5 year survival rate approaches 85% in patients with FHF after they receive a liver transplant (Asher et al, 1993; Wei et al, 1997).

Although researchers have described the clinical course of fulminant hepatic failure in detail, very little is known regarding the pathophysiology of the syndrome on the cellular level in humans. Most studies looking at biochemical abnormalities of the liver have been conducted on animals (Newsome et al, 2000). Most studies have looked at indices of liver necrosis and poor synthetic function and very few have any data on abnormalities of metabolic pathways on a cellular level in the liver (Blitzer et al, 1978; Francavilla et al, 1989; Sielaff et al, 1995; Diaz-Buxo et al, 1997; Kalpana et al, 1999). Some human studies have looked at abnormalities of the brain cells in the context of fulminant hepatic failure (Strauss et al; 1997, Clemmesen et al, 1999).

Most clinicians are aware that, in humans, glucose levels are low early in the syndrome and are correcting the hypoglycaemia with glucose infusions. There is also evidence that lactate is high in severe disease (Jalan et al, 1999). There is therefore indirect evidence that glycolysis is not efficient and ATP is produced anaerobically. There are no studies investigating the time course of those abnormalities and there are no data regarding the performance of other key metabolic pathways in the liver. As we know from clinical experience, there is progressive deterioration of liver function

during the course of the disease and we can assume that many metabolic pathways are affected as the disease evolves.

The aim of our study was to identify abnormalities in key metabolic pathways in patients with fulminant hepatic failure. We compared key metabolite concentrations between patients and controls using ^{1}H NMR Spectroscopy. I n a subsequent subgroup analysis we compared concentrations of those metabolites between severely affected individuals and moderately affected individuals. Patients were divided from the beginning in two groups: those with POD and those of other aetiologies. We also studied the evolution in time concentrations of key metabolites during the early stages of fulminant hepatic failure in both groups.

7.2 MATERIALS AND METHODS

7.2.1` PATIENTS.

The study was conducted between May 96 and July 99. We studied 34 patients with fulminant hepatic failure caused by paracetamol overdose. We also studied 34 patients with fulminant hepatic failure caused by other aetiologies. The aetiologies for the fulminant hepatic failure in those patients are shown in Figure 7.1. All patients were admitted and cared for at the Scottish Liver Transplant Unit and the Intensive Care Unit at the Royal Infirmary of Edinmburgh. All patients were encephalopathic and they presented with an International Normalised Ratio (INR) of more than 3.0. Individual characteristics of the patients in the two groups are shown in Table 7.1 Our control group consisted of 17 healthy volunteers (10 males, 7 fermales,mean age (39.8 ± 7.3)) .

Blood was collected on admission a means of 5.4 hours post admission (range 2 -11 hours) for the Day 1 analysis and a means of 52.6 hours (48- 69) for the Day 3 analysis, in lithium heparin tubes. Blood from volunteers was collected a mean of 2 hours post prandially as the differences in amino acid concentrations between sexes are minimal at that time (Morgan et al, 1982). It was immediately centrifuged at 2000 g for 15 minutes at 4^0C. The supernatant was then pippetted and aliquoted in 2.5 ml vials. The vials were then stored at -40^0C until NMR analysis.

Table 7.1

Individual characteristics of patients with fulminant hepatic failure

	NON POD FHF	POD FHF
AGE	50.7 ± 9.3 yrs	38.7 ± 6.4 yrs
MALES	13	11
FEMALES	21	23
TIME FROM ONSET OF SYMPTOMS TO ADMISSION TO SLTU	8.6 ± 4.7 days	3.2 ± 1.8 days

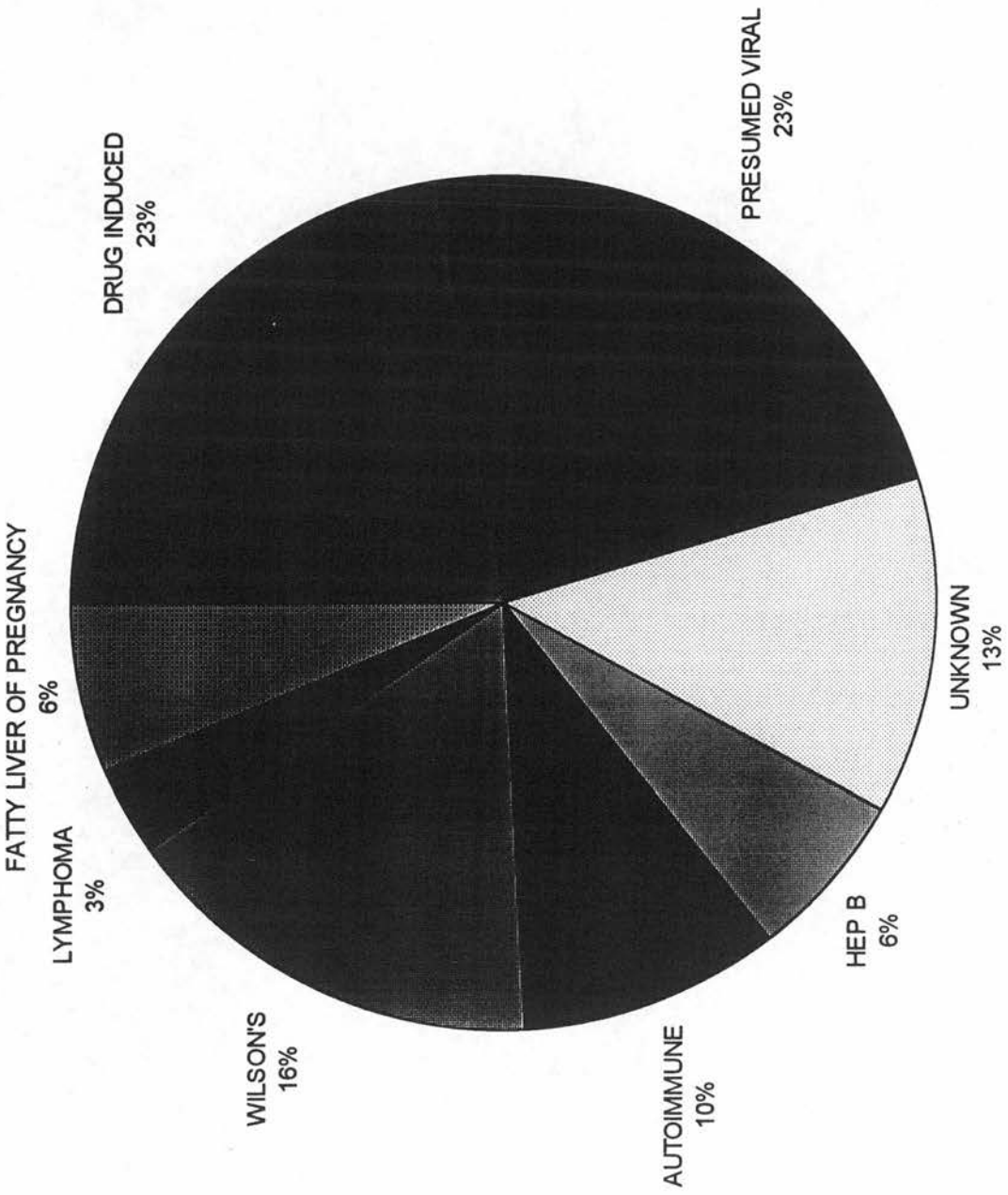


Figure 7.1 Aetiologies of non paracetamol fulminant hepatic failure in the study population

7.2.2 Sample preparation for NMR spectroscopy

Samples were prepared by adding a D_2O solution of (150 µl) to plasma (600 µl) providing thus an internal field frequency lock for the spectrometer. Chemical shifts were referenced externally to the singlet methyl resonance of Sodium 3-(trimethylsilyl 2,2,3,3-$^2H_4$) -1 propionate (TSP) at zero ppm.

7.2.3 NMR Spectroscopy monitoring

We measured the concentration of the following substances in the plasma using NMR spectroscopy: Acetate, acetoacetate and ß- hydroxybutyrate as they are the main ketone bodies mainly exported by liver cells (Fuchs at al, 1994; Duee et al, 1994; Gerlach et al, 1996), leucine, isoleucine, phenylalanine and tyrosine consumption as they are the key ketogenic amino acids used by the hepatocyte (Gerlach et al, 1996), glucose, alanine and threonine consumption from plasma as a measure of glycolysis to monitor the cells energy requirements (Seglen, 1974; Sefter et al, 1994), lactate and pyruvate as a measure of the active aerobic and anaerobic glycolysis (Seglen, 1974), the consumption of valine, isoleucine and methionine as succinate formation precursors (De Blaauw et al, 1998) and the concentrations of glutamate, glutamine, histidine and arginine which are indices of active transamination and urea synthesis (Lang et al, 1990; De Blaauw et al, 1998). Concentrations of glycerol were also measured as evidence of on going fatty acid oxidation. We have also measured the concentrations of methylamine, dimethylamine, trimethylamine and TMAO as they are thought to be produced by acute injury to hepatocytes. Finally, myoinositol an essential osmotic buffer was measured as well.

7.2.4 Proton NMR spectroscopy

NMR Spectroscopy monitoring was performed as discussed in Chapter 2. Sample preparation, data acquisition and quantitation of compound concentrations were performed as discussed in Chapter 2. Instead of the presaturation technique to acquire our data, the CMPG sequence was applied, as this sequence enabled us to observe a flat baseline in our spectra from plasma samples, by minimising the signals acquired from macromolecules present in the plasma like proteins and lipoproteins.

Figure 7.2 shows a 1H NMR spectrum from plasma from a normal volunteer and Figure 7.3 shows a 1H NMR spectrum from plasma of a patient suffering from fulminant hepatic failure.

7.2.5 Statistical analysis

Separate analysis was performed for fulminant hepatic failure from paracetamol overdoses and the fulminant hepatic failure caused by other aetiologies. To compare between groups we used the Student's t-Test for parameters with non-missing values and the Mann Witney U test for parameters with missing values. Values are expressed as mean (range and Standard error). A p value of<0.05 was taken as statistically significant (two- tail test of significance).

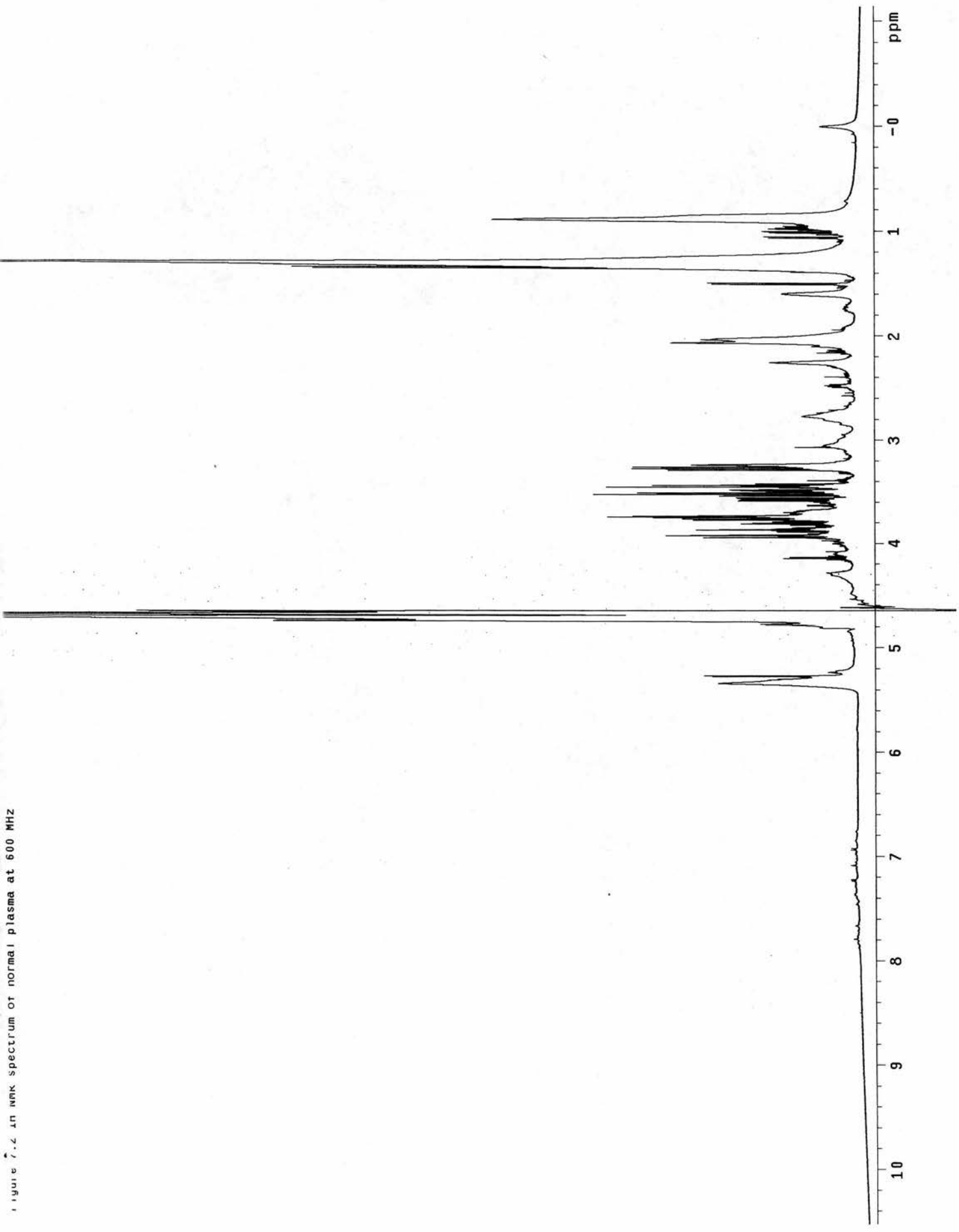


Figure 7.2 1H NMR spectrum of normal plasma at 600 MHz

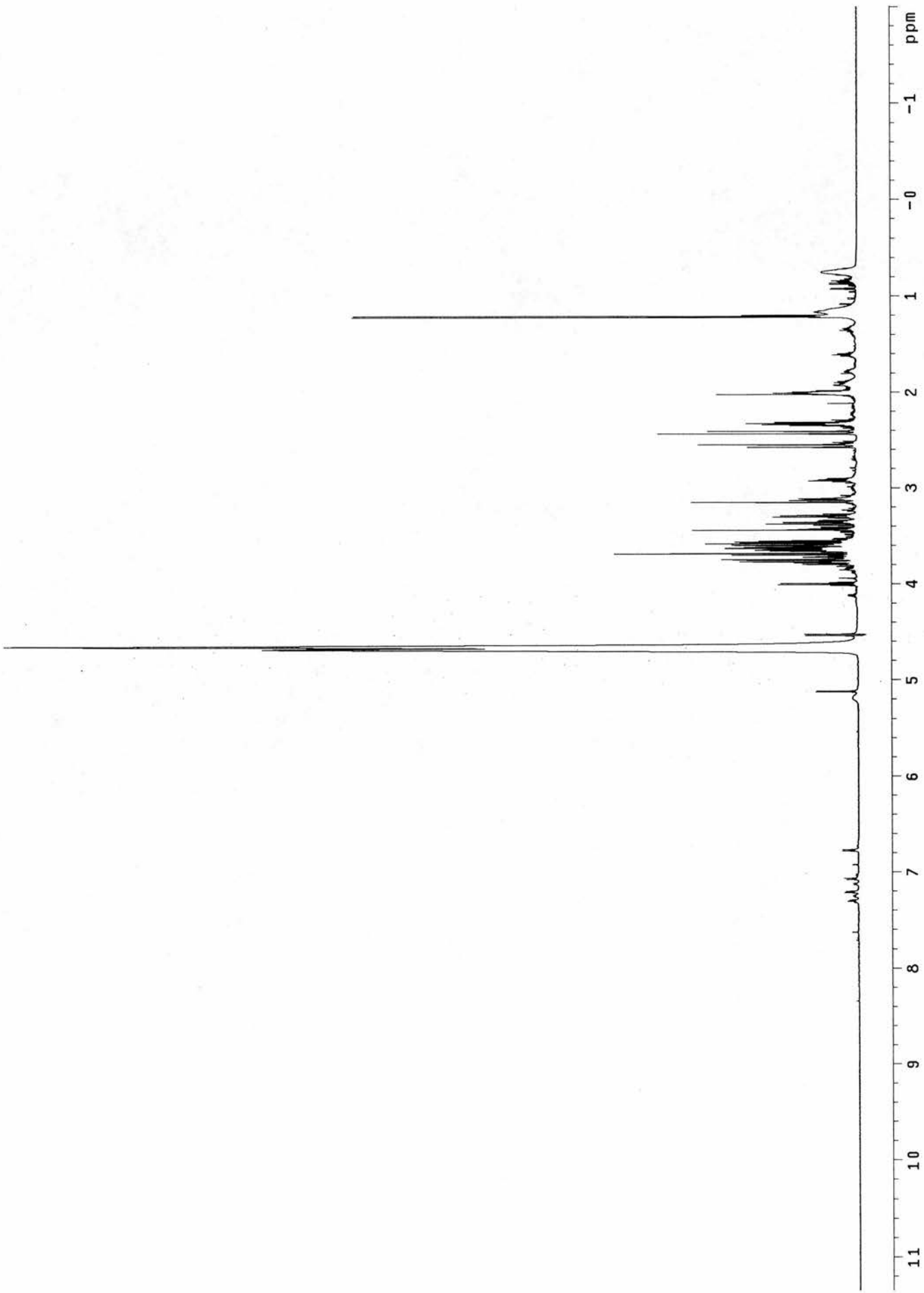


Figure 7.3 1H NMR spectrum from plasma of a patient with fulminant hepatic failure at 600 MHz

7.3 RESULTS

7.3.1 Patients vs controls

We studied patients with fulminant hepatic failure due to aetiologies other than paracetamol overdose (group A, 34 patients), patients with fulminant hepatic failure due to paracetamol overdose (group B, 34 patients) and sex and age matched normal controls (group C, 17subjects). There was evidence of significant dysfunction of the major metabolic pathways both in patients whose aetiology was paracetamol overdose and in patients whose aetiology was not paracetamol overdose compared to normal controls. We will look at those pathways in detail:

7.3.1.1 GLYCOLYSIS AND GLYCONEOGENESIS

Due to continuous intravenous infusion of glucose it was impossible to measure directly the concentrations of glucose in patients. The results for lactate and pyruvate are shown on Figure 7.4. Lactate concentrations in group A were higher than group B and the results were highly significant statistically ($p<0.0000$). Lactate concentrations in group C were also higher with a highly significant statistical result($p<0.0000$).

Pyruvate concentrations in Group A were higher than controls and the results were statistically significant ($p<0.0005$). Also higher than controls were pyruvate concentrations in Group B and the results were statistically significant ($p<0.018$).

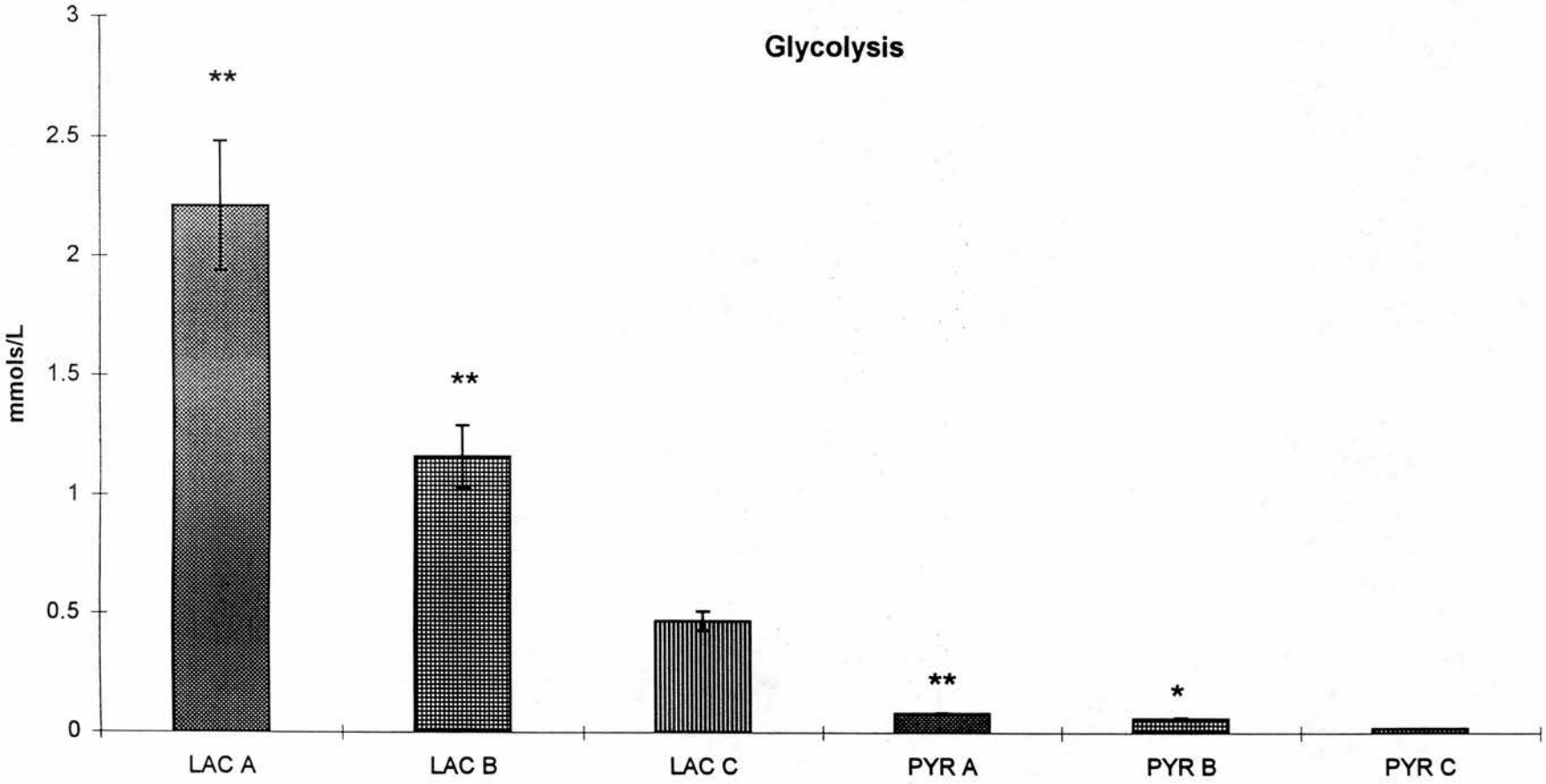
We have also looked at four glucogenic amino acids. The overall results for those are shown on Figure 7.5. Alanine which can be converted to pyruvate, threonine and glycine which are precursors of alanine and aspartate which can be

converted to phosphoenolpyruvate. Concentrations of alanine were higher in patients than controls. If we compare group A with group C , the results were statistically significant ($p<0.014$) and so were the results of the comparison between group B and group C ($p<0.004$).

Concentrations of glycine were higher in patients than controls. If we compare group A with group C, the results were statistically highly significant ($p<0.0000$). If we compare group B with group C, the results were statistically highly significant ($p<0.00001$).

There were no significant differences in the concentrations of threonine comparing patients and controls. Concentrations of aspartate were higher in patients than controls. No aspartate was detected in controls. If we compare group A with group C, the results were highly significant ($p<0.00007$). If we compare group B with group C, the results were highly statistically significant ($p<0.00004$).

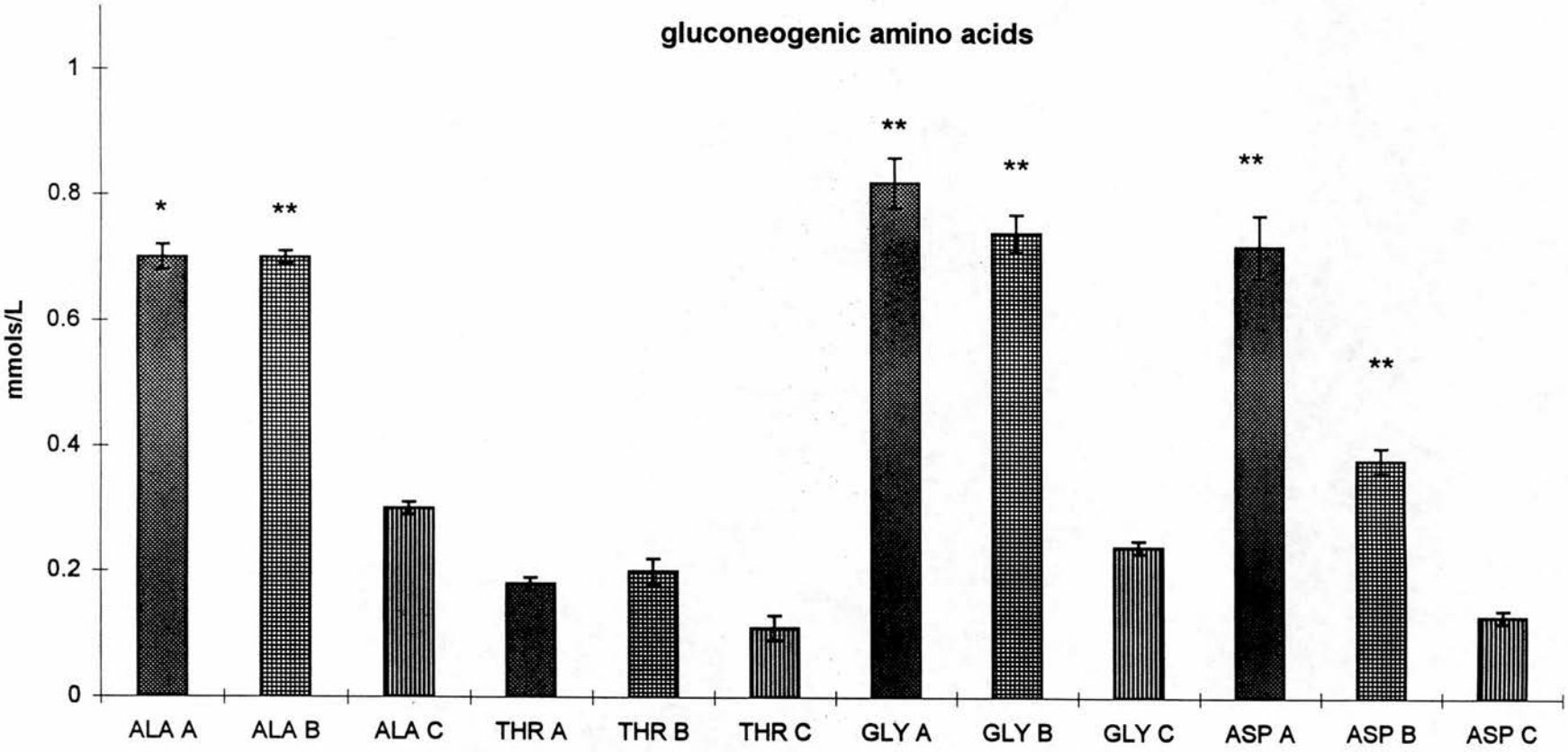
FIGURE 7.4



Concentrations of lactate (LAC) and pyruvate (PYR) in plasma of patients with non-POD FHF (A) with POD FHF (B) and controls C

Results shown are means and the error bars represent SEM. Comparisons are made between controls and the two groups of patients. *p<0.05 ** p<0.01

FIGURE 7.5



Concentrations of gluconeogenic amino acids Alanine (ALA), Treonine (THR), Glycine (GLY) and Aspartate (ASP) in plasma of patients with non-POD FHF (A) with POD FHF (B) and controls C

Results shown are means and the error bars represent SEM. Comparisons are made between controls and the two groups of patients. *p<0.05 ** p<0.01

7.3.1.2 BRANCH CHAIN AMINO ACIDS

The overall results for the branch chain amino acids are shown in Figure 7.6. There were no statistically significant differences in the leucine concentrations between patients and controls

Overall concentrations of isoleucine were higher in patients than controls. If we compare group A with group C, the results were statistically significant ($p<0.007$). If we compare group B with group C, the results were also statistically significant ($p<0.013$).

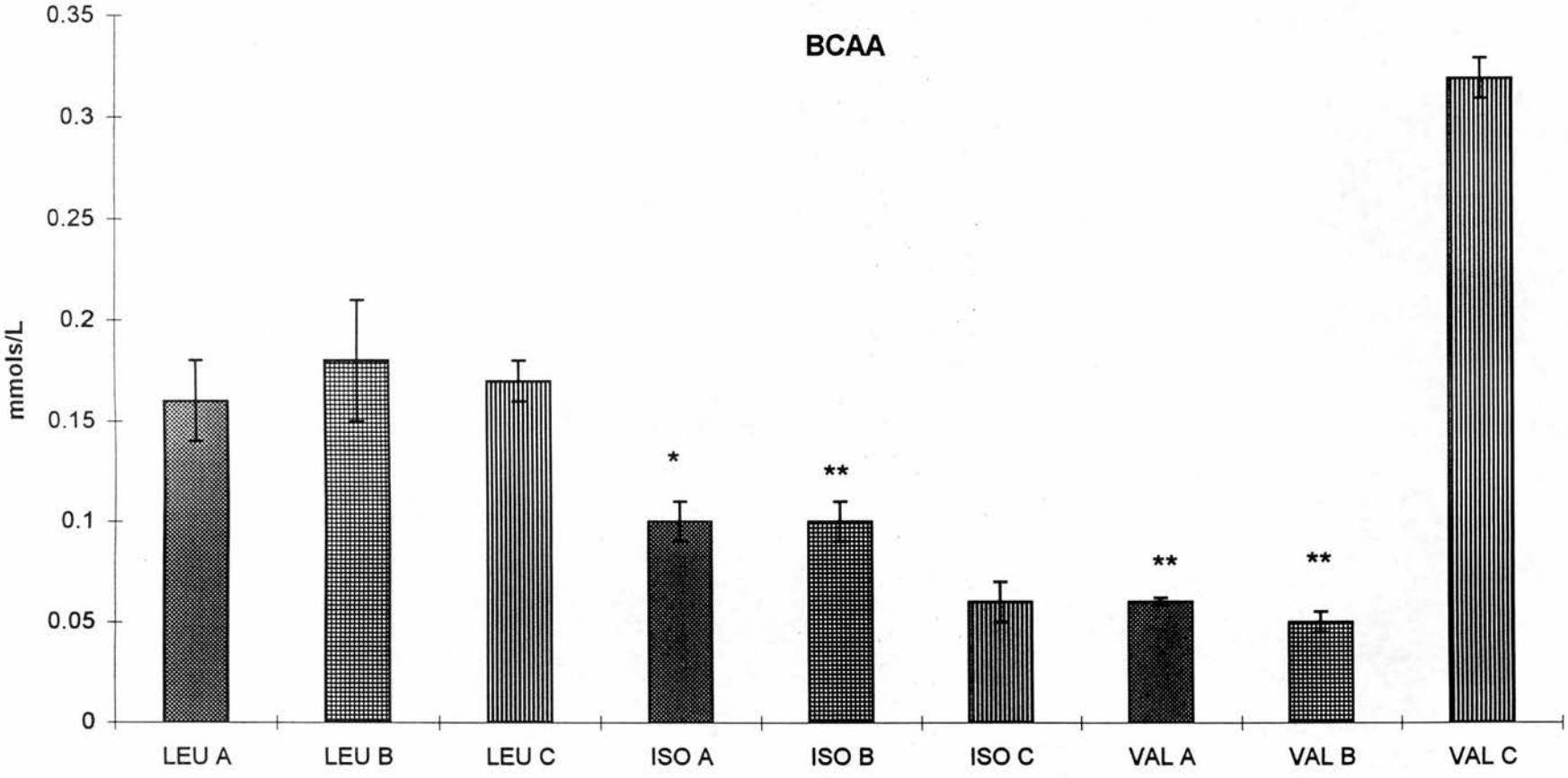
The concentrations of valine were lower in patients than controls. If we compare group A with group C, the results were highly significant ($p<0.0000$). If we also compare group B with group C , the results were again highly statistically significant ($p<0.0000$).

7.3.1.3 AROMATIC AMINO ACIDS

Overall results for the aromatic amino acids and methionine are shown in Figure 7.7. Concentrations of tyrosine were higher in patients than controls. If we compare group A with group C, the results were statistically highly significant ($p<0.0001$). If we compare group B with group C, the results were statistically highly significant ($p<0.000$). Phenylalanine concentrations which is a tyrosine precursor, were also measured and were found to be higher in patients than controls. If we compare group A with group C, the results were statistically significant ($p<0.0002$). If we compare group B with group C, the results were statistically highly significant ($p<0.0000$).

The concentrations of methionine were higher in patients than controls. In fact no methionine was detected in any of the samples from the controls. If we compare group A with group C, the results were statistically significant ($p<0.003$). If we compare group B with group C, the results were statistically significant($p<0.016$).

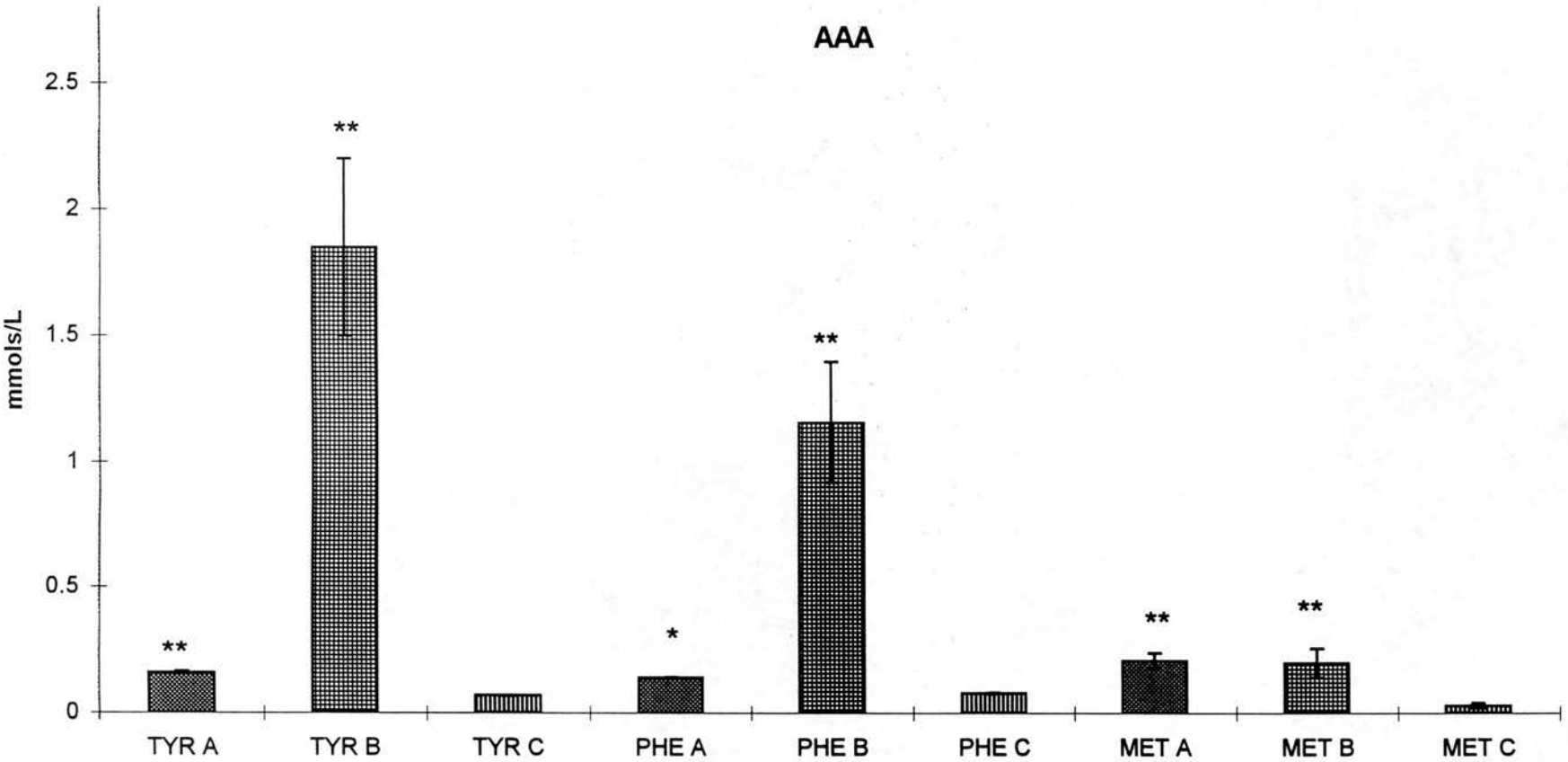
FIGURE 7.6



Concentrations of branch chain amino acids Leucine (LEU), Isoleucine (ISO) and Valine (VAL) in plasma of patients with non-POD FHF (A) with POD FHF (B) and controls C

Results shown are means and the error bars represent SEM. Comparisons are made between controls and the two groups of patients. *p<0.05 ** p<0.01

FIGURE 7.7



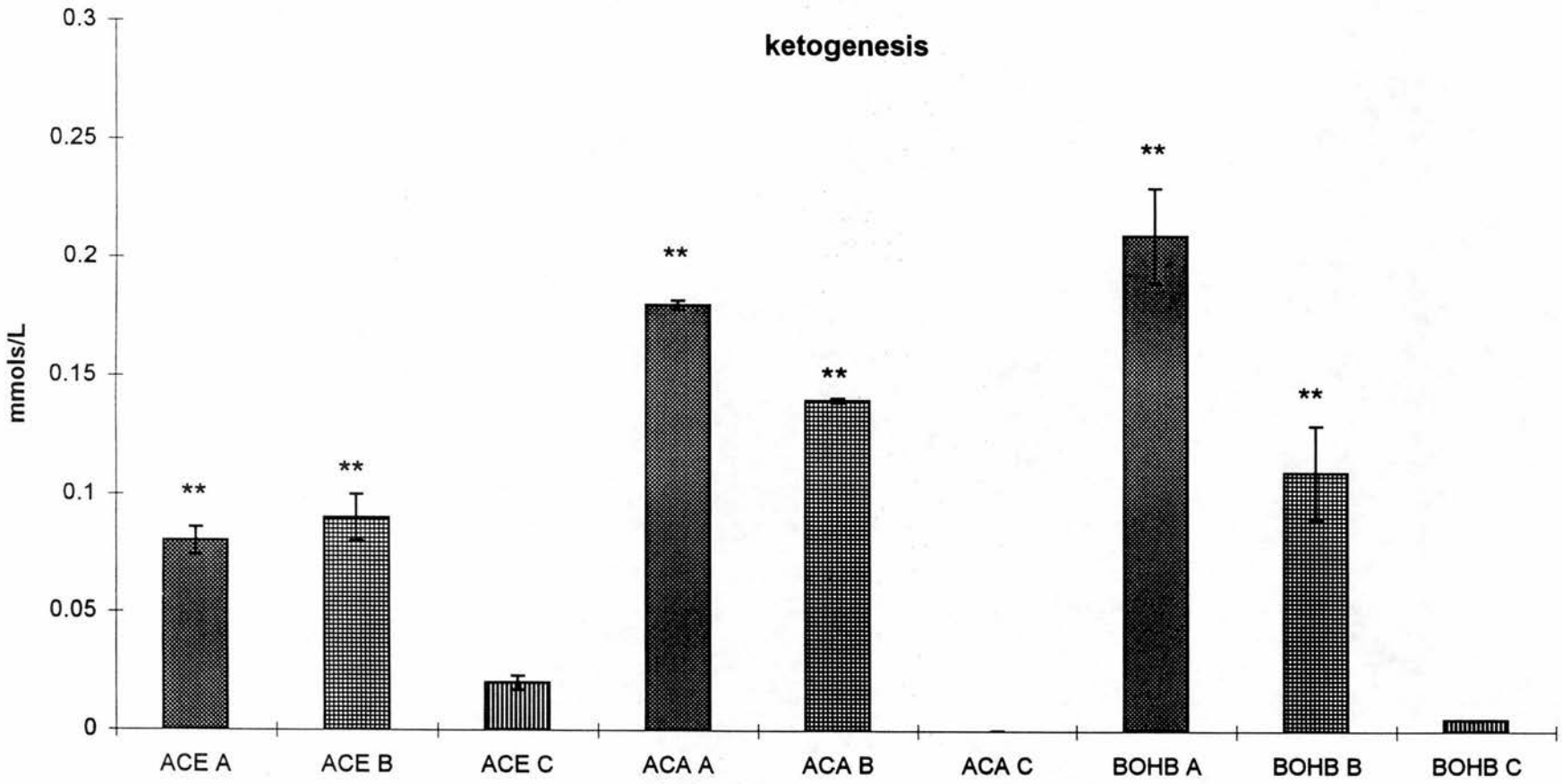
Concentrations of aromatic amino acids Tyrosine (TYR) and Phenylalanine (PHE) and Methionine (MET) in plasma of patients with non-POD FHF (A) with POD FHF (B) and controls C.

Results shown are means and the error bars represent SEM. Comparisons are made between controls and the two groups of patients. *p<0.05 ** p<0.01

7.3.1.4 KETOGENESIS

1H NMR Spectroscopy gives us the opportunity to look at ketone bodies formed in the process of ketogenesis. The abundant ketone bodies in the human body are acetoacetate, acetate and hydroxybutyrate. They are formed in the liver as a result of fatty acid oxidation. Overall results for ketone bodies are shown in Figure 7.8. For all three, concentrations in patients were higher than concentrations in controls. For acetoacetate, if we compare group A with group C, the results were statistically highly significant ($p<0.0004$). If we compare group B with group C, the results were statistically highly significant ($p<0.0003$). For acetate, if we compare group A with group C, the results were statistically significant ($p<0.007$). If we compare group B with group C, the results were statistically significant ($p<0.004$). For hydroxybutyrate, if we compare group A with group C, the results were statistically highly significant ($p<0.0000$). If we compare group B with group C, the results were statistically significant ($p<0.009$).

FIGURE 7.8



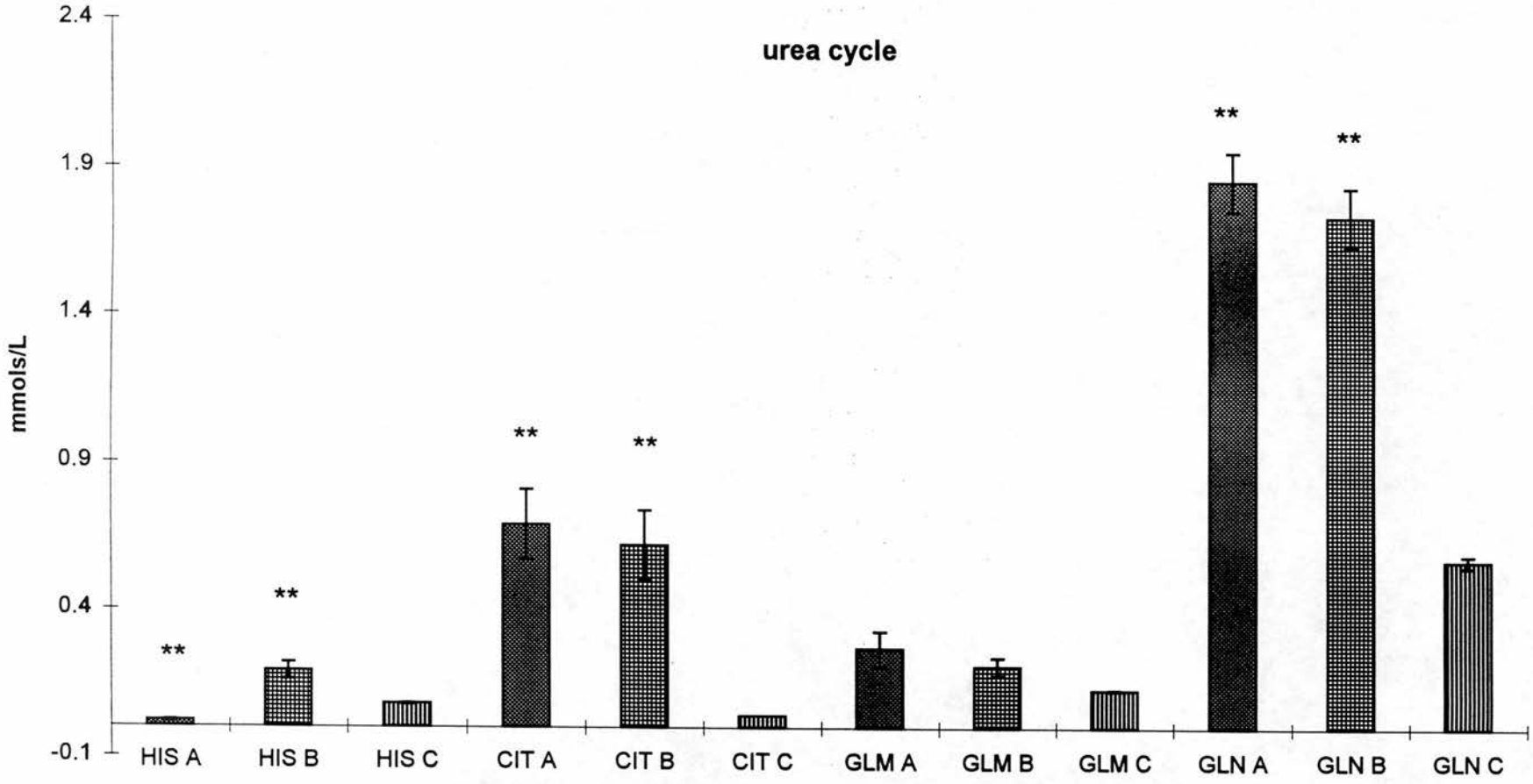
Cponcentrations of ketone bodies Acetate (ACE), Acetoacetate (ACA) and B-hydroxybutyrate (BOHB)in plasma of patients with non-POD FHF (A), POD FHF (B) and controls (C)

Results shown are means and the error bars represent SEM. Comparisons are made between controls and the two groups of patients. *p<0.05 ** p<0.01

7.3.1.5 UREA AND GLUTAMINE SYNTHESIS

Urea synthesis is vital in order to get rid of unwanted nitrogen. The urea cycle takes place exclusively into the liver parenchymal cells and is fed by histidine which is converted to glutamate, by glutamate itself, and aspartate. By 1H NMR Spectroscopy we were able to detect all those urea cycle precursors in the plasma of patients along with arginine and citrulline two amino acids which are directly implicated in the urea cycle and glutamine which can be an end product for the metabolism of glutamate. Overall results for these metabolites are shown in Figure 7.9. Looking at histidine concentrations in detail they were highest in patients suffering fulminant hepatic failure from paracetamol overdose (Group B), followed by controls (group C) and the lowest concentrations were observed in patients suffering fulminant hepatic failure from other aetiologies (Group A). If we compare group A with group C, the results were statistically highly significant ($p<0.0000$). If we compare group B with group C, the results were statistically significant ($p<0.0006$). There were no significant differences in the concentrations of glutamate, if we compared patients and controls. There were no significant differences in the concentrations of arginine, comparing patients and controls. Concentrations of citrulline were higher in patients than controls. If we compare group A with group C, the results were highly significant ($p<0.0000$). If we compare group B with group C, the results were highly statistically significant ($p<0.00003$). Also concentrations of glutamine were higher in patients than controls. If we compare group A with group C, the results were statistically significant ($p<0.0005$). If we compare group B with group C, the results were statistically significant ($p<0.002$).

FIGURE 7.9



Concentrations of urea synthesis intermediates, Histidine (HIS), Citrulline (CIT) and Glutamate (GLM) and Glutamine (GLN) in plasma of patients with non-POD FHF (A) with POD FHF (B) and controls C.

Results shown are means and the error bars represent SEM. Comparisons are made between controls and the two groups of patients. *p<0.05 ** p<0.01

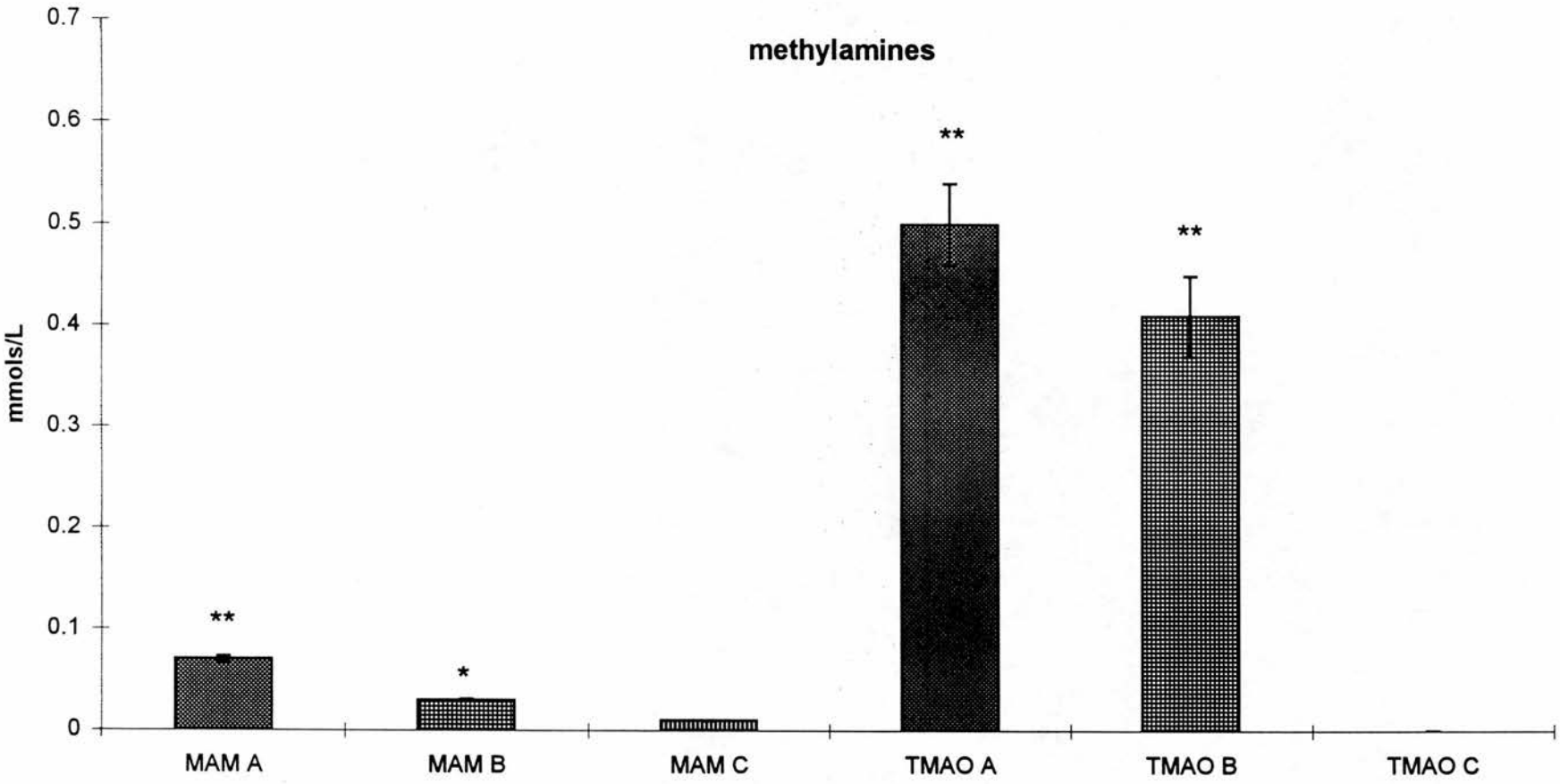
7.3.1.6 METHYLAMINES

It was postulated that the production of methylamines is high in the instance of fulminant hepatic failure. It was thought that this was a way for the body to metabolise nitrogen which can be toxic in the instance of a non - functioning urea cycle. Using 1H NMR Spectroscopy we have looked at the production and concentrations of methylamine, dimethylamine, trimethylamine and trimethylamine oxide (TMAO). Overall results for the methylamines that we have detected are shown in Figure 7.10. Concentrations of methylamine were higher in patients than controls. If we compare group A with group C, the results were statistically significant ($p<0.0002$). If we compare group B with group C, the results were statistically significant ($p<0.012$). There were no significant differences in the concentrations of dimethylamine or trimethylamine, comparing patients and controls. Concentrations of TMAO were higher in patients than controls. In fact, no TMAO was detected in the plasma of control subjects. If we compare group A with group C, the results were highly significant ($p< 0.0000$). If we compare group B with group C, the results were highly statistically significant ($p<0.0000$).

7.3.1.7 GLYCEROL AND MYOINOSITOL

We have also looked at the concentrations of those two substances. Overall results are shown in Figure 7.11 for those two substances. Glycerol as a marker of fatty acid oxidation and myoinositol as a marker of osmotic abnormalities in the living cells. No statistically significant differences were observed in the concentrations of glycerol or myoinositol between patients and controls.

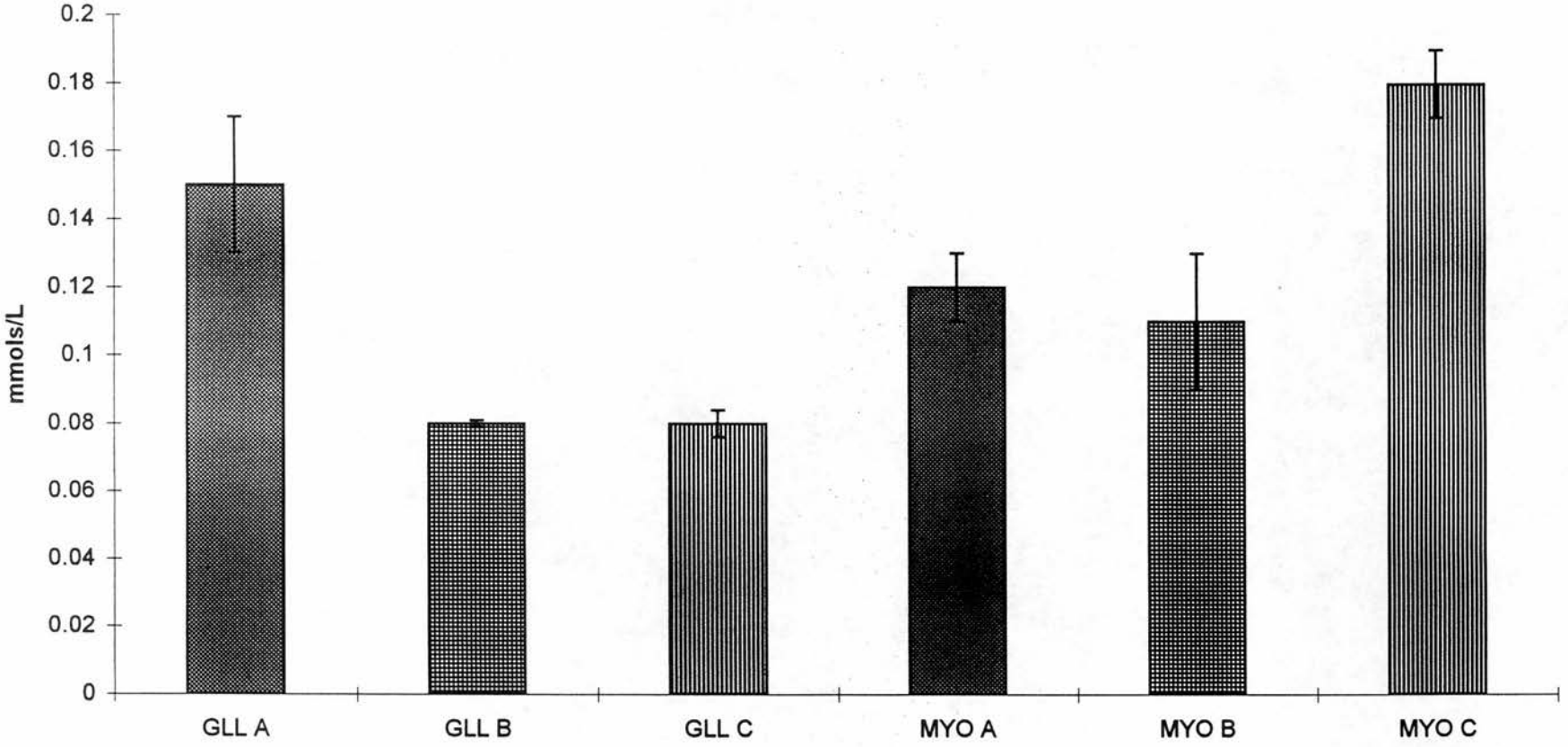
FIGURE 7.10



Concentrations of methylamine (MAM) and TMAO in plasma of patients with non-POD FHF (A) with POD FHF (B) and controls C.

Results shown are means and the error bars represent SEM. Comparisons are made between controls and the two groups of patients. *p<0.05 ** p<0.01

FIGURE 7.11



Concentrations of glycerol (GLL) and myoinositol (MYO) in plasma of patients with non-POD FHF (A) with POD FHF (B) and controls C

Results shown are means and the error bars represent SEM. Comparisons are made between controls and the two groups of patients.

7.3.2 SEVERELY VS MODERATELY AFFECTED PATIENTS

7.3.3 NON-POD FHF.

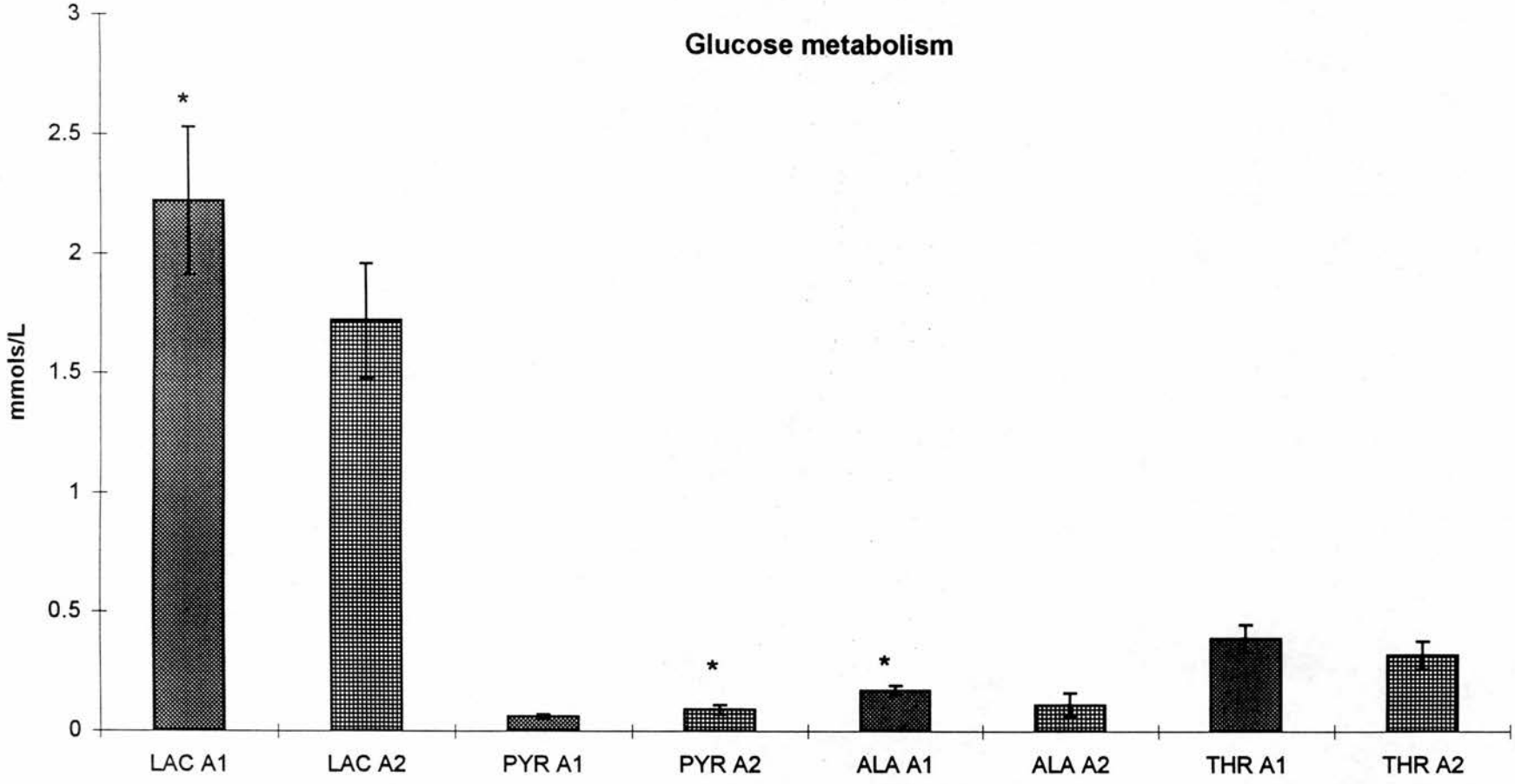
In total 20 patients with non -POD FHF died or were transplanted. As those are events that abruptly terminate the disease (censored events) for the purpose of analysis they constituted Group A1. Fourteen patients survived with maximal supportive therapy and they did not undergo orthotopic liver tranplantation. For the purpose of analysis they constituted Group A2.

We looked at the following key metabolic pathways in detail.

7.3.3.1 GLYCOLYSIS AND GLUCONEOGENESIS.

Results concerning the performance of the glycolytic pathway are shown in Figure 7.12 for Day 1 and in Figure 7.13 in Day 3. There were statistically significant differences between the two groups both on Day1 and on Day 3. On day 1, lactate was significantly higher in Group A1 ($p<0.023$). Pyruvate was significantly lower in Group A1 ($p<0.014$). Alanine a glucogenic amino acid was significantly higher in Group A1 ($p<0.032$). On day 3, lactate was significantly higher in Group A1 ($p<0.002$). Pyruvate was significantly lower in Group A1 ($p<0.0078$). Alanine was significantly higher in Group A1 ($p<0.011$). No statistically significant differences were observed in the concentrations of Threonine, in either Day 1 or Day 3.

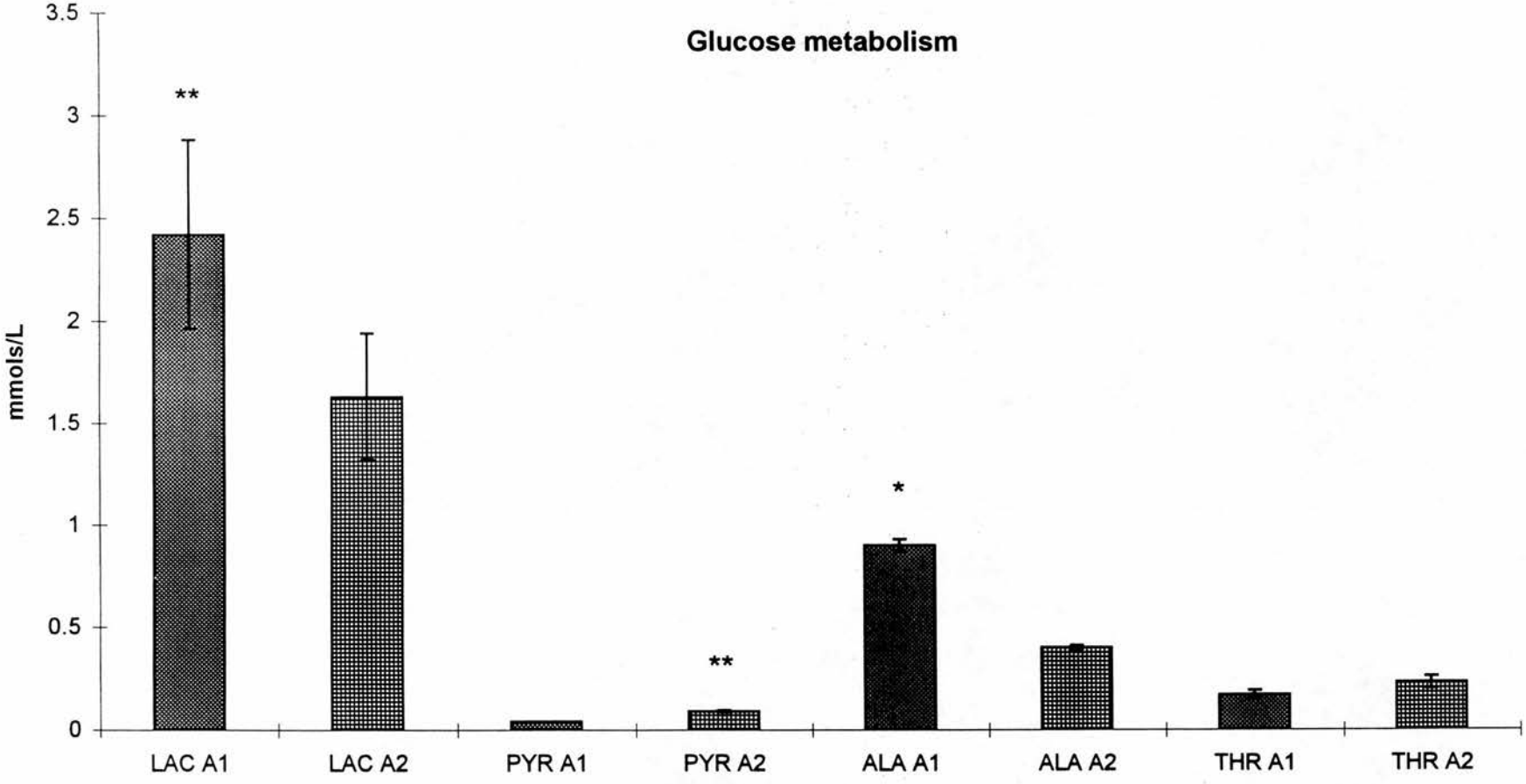
FIGURE 7.12



Concentrations of the intermediates of glucose metabolism in non-POD FHF on day 1 after admission

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

FIGURE 7.13



Concentrations of the intermediates of glucose metabolism in non-POD FHF on day 3 after admission.

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05 ** p<0.01

7.3.3.2 BCAA

Results concerning the branch chain amino acids are shown in Figure 7.14 for Day 1 and in Figure 7.15 in Day 3. No difference was observed on Day 1 between concentrations of leucine. On Day 3, there was a statistically significant difference observed between the concentrations of leucine, one of the ketogenic amino acids, which was significantly lower in severely affected patients ($p<0.023$). Valine was significantly higher in Group A1 on Day 1. ($p<0.022$). Valine was significantly higher in Group A1 also on Day 3. ($p<0.039$). There were no differences in the concentration of isoleucine either on Day 1 or on Day 3.

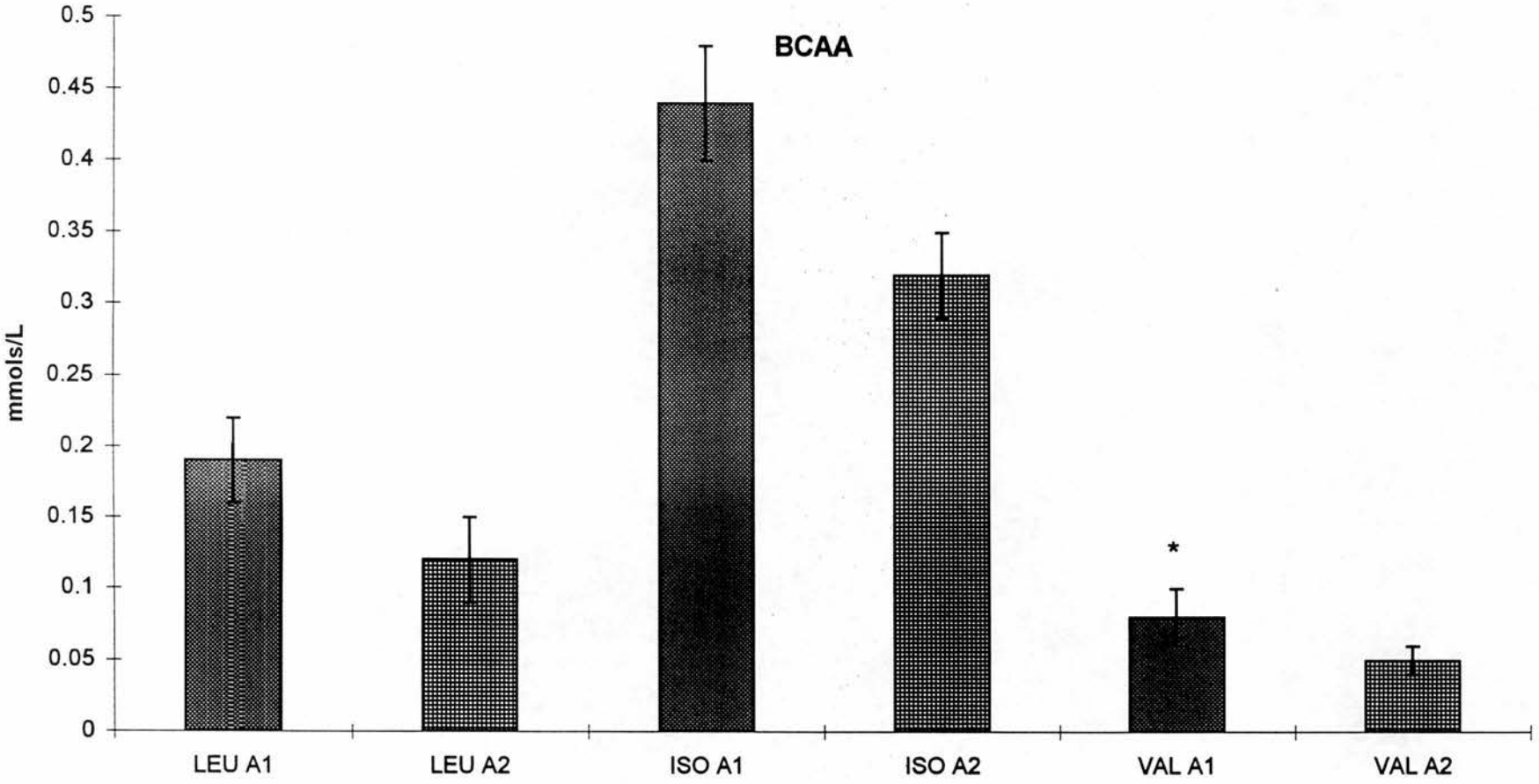
7.3.3.3 AAA

There were no differences in the concentration of the aromatic amino acids Phenylalanine and Tyrosine either on Day 1 or on Day 3. There were no differences in the concentration of methionine either on Day 1 or on Day 3.

7.3.3.4 KETOGENESIS

There were no statistically significant differences on Day 1 concerning the concentrations of acetoacetate, ß-hydroxybutyrate and acetate. Results concerning the performance of the ketogenic pathway are shown on Figure 7.16 for Day 3. By Day 3, there were statistically significant differences in the concentrations of acetoacetate ($p<0.007$) and acetate ($p<0.017$). Both were higher in Group A1. There were no differences in the concentrations of ß-hydroxybutyrate.

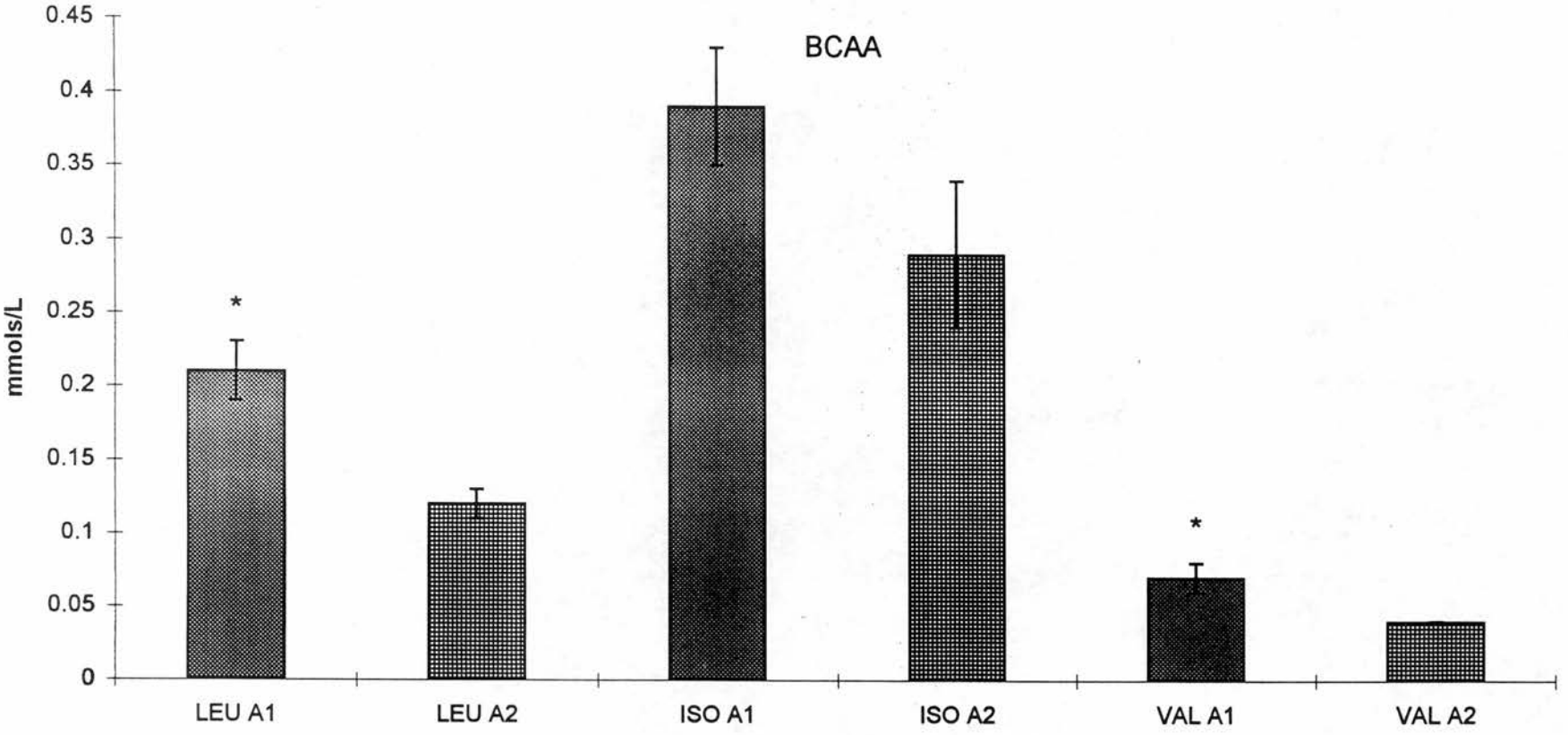
FIGURE 7.14



Concentrations of branch chain amino acids in non-POD FHF on day 1 after admission.

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

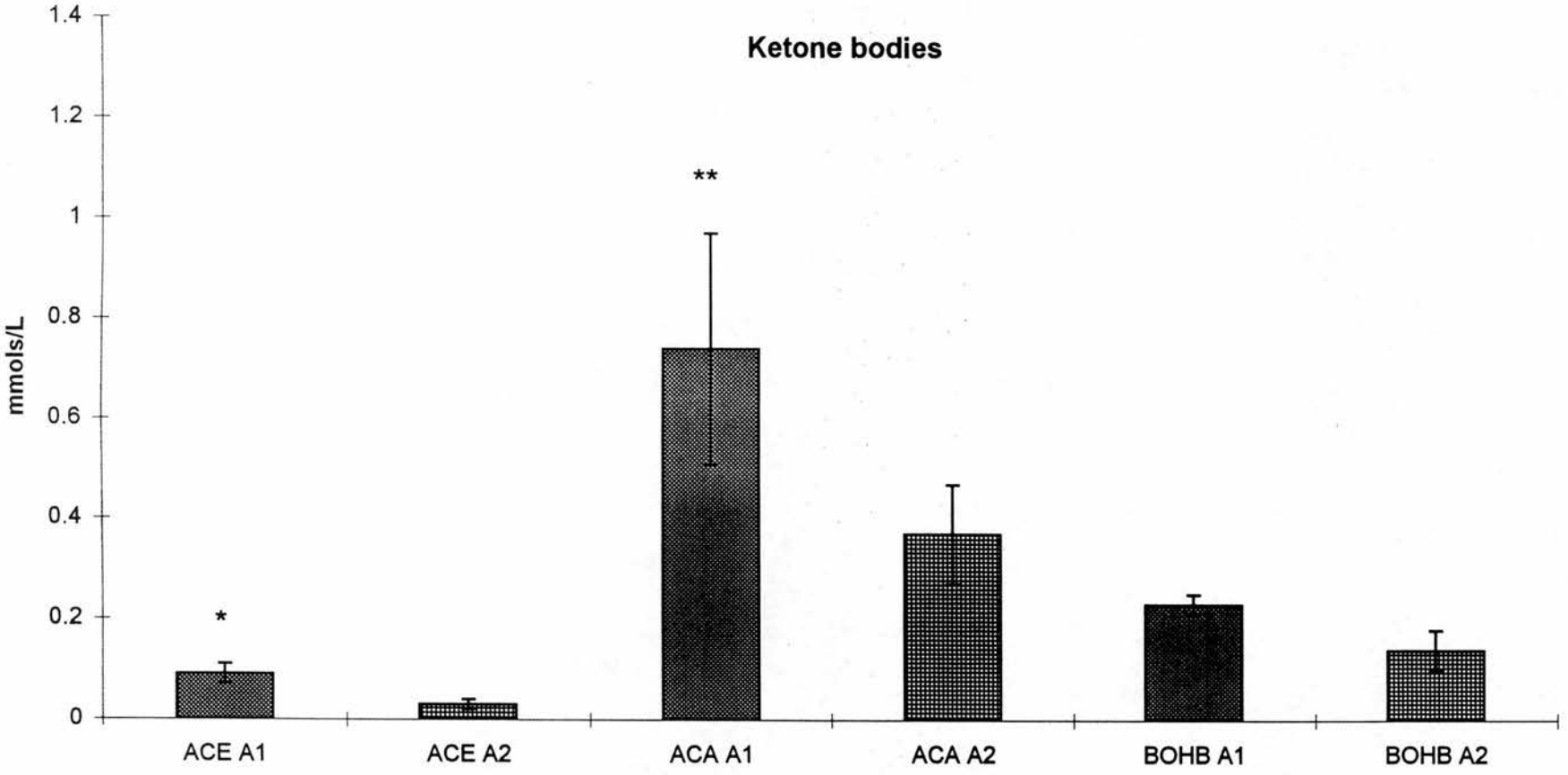
FIGURE 7.15



Concentrations of branch chain amino acids in non-POD FHF on day 3 after admission

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

FIGURE 7.16



Concentrations of ketone bodies in non-POD FHF on day 3 after admission.

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05 ** p<0.01

7.3.3.5 UREA AND GLUTAMINE SYNTHESIS.

The fate of metabolism of nitrogen containing compounds, was the same in the two groups. There were no statistically significant differences in the concentration of histidine, glutamate, aspartate or arginine, between the two Groups on both days. There was a statistically significant difference in the concentration of citrulline on day 1 ($p<0.036$) and on Day 3 ($p<0.022$). Citrulline production was higher in Group A2 on both days. Glutamine concentrations were similar in both groups in both days

7.3.3.6 METHYLAMINES PRODUCTION.

We did not find any differences in the production of those substances between the two groups. There were no statistically significant differences in the concentration of Methylamine, Dimethylamine, Trimethylamine and TMAO, on Day 1 and Day 3

No differences were elicited in the production of glycerol and myoinositol on both day 1 and Day 3.

7.3.4 POD FHF

In total 19 patients with POD FHF died or were transplanted. As those are censored events for the purpose of analysis they constituted Group B1. Fifteen patients survived with maximal supportive therapy and they did not undergo orthotopic liver tranplantation. For the purpose of analysis they constituted Group B2. We looked at the following key metabolic pathways in detail.

7.3.4.1 GLYCOLYSIS AND GLUCONEOGENESIS.

Results concerning the performance of the glycolytic pathway are shown in Figure 7.17 for Day 1 and in Figure 7.18 in Day 3. There were statistically significant differences between the two groups both on Day1 and on Day 3. On day 1, lactate was significantly higher in Group B1 ($p<0.044$). Pyruvate was significantly higher in Group B1 ($p<0.03$). Alanine a glucogenic amino acid was significantly higher in Group B1 ($p<0.048$). On day 3, lactate was significantly higher in Group B1($p<0.016$). Pyruvate was significantly higher in Group B1 ($p<0.013$). There were no statistically significant differences in the concentration of Alanine on Day 3. No statistically significant differences were observed in the concentrations of Threonine and Methionine.

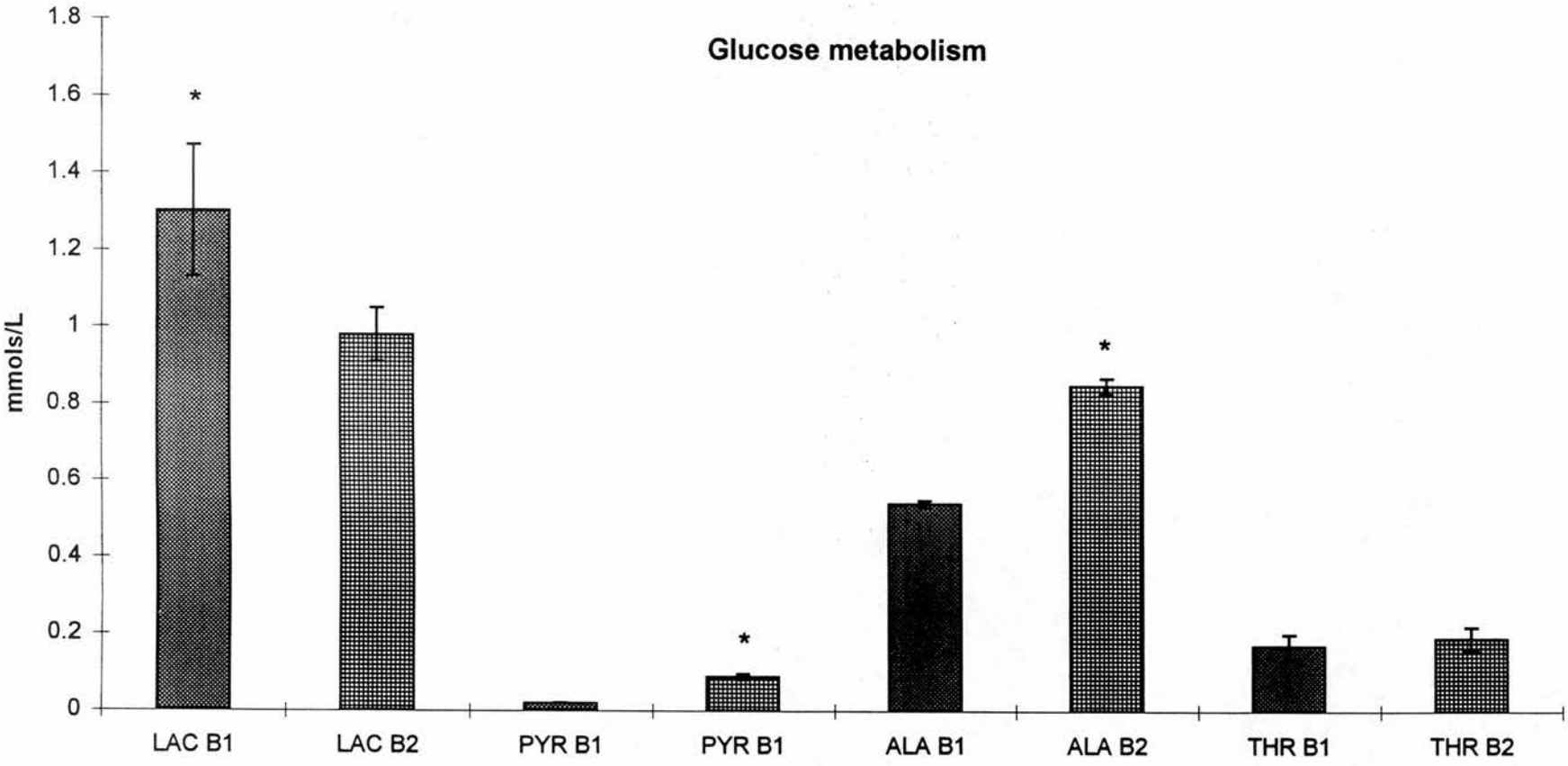
7.3.4.2 BCAA

There were no differences in the concentration of the branch chain amino acids Leucine, Isoleucine and Valine either on Day 1 or on Day 3.

7.3.4.3 AAA

Results concerning the branch chain amino acids are shown in Figure 7.19 for Day 1 and in Figure 7.20 in Day 3. There were statistically significant differences in the concentration of phenylalanine on Day 1 ($p<0.021$). Statistically significant differences were also shown in the concentration of phenylalanine on Day 3 ($p<0.011$). On both occasions phenylalanine concentrations were higher on Group B2. There were no differences in the concentrations of tyrosine or methionine on either days.

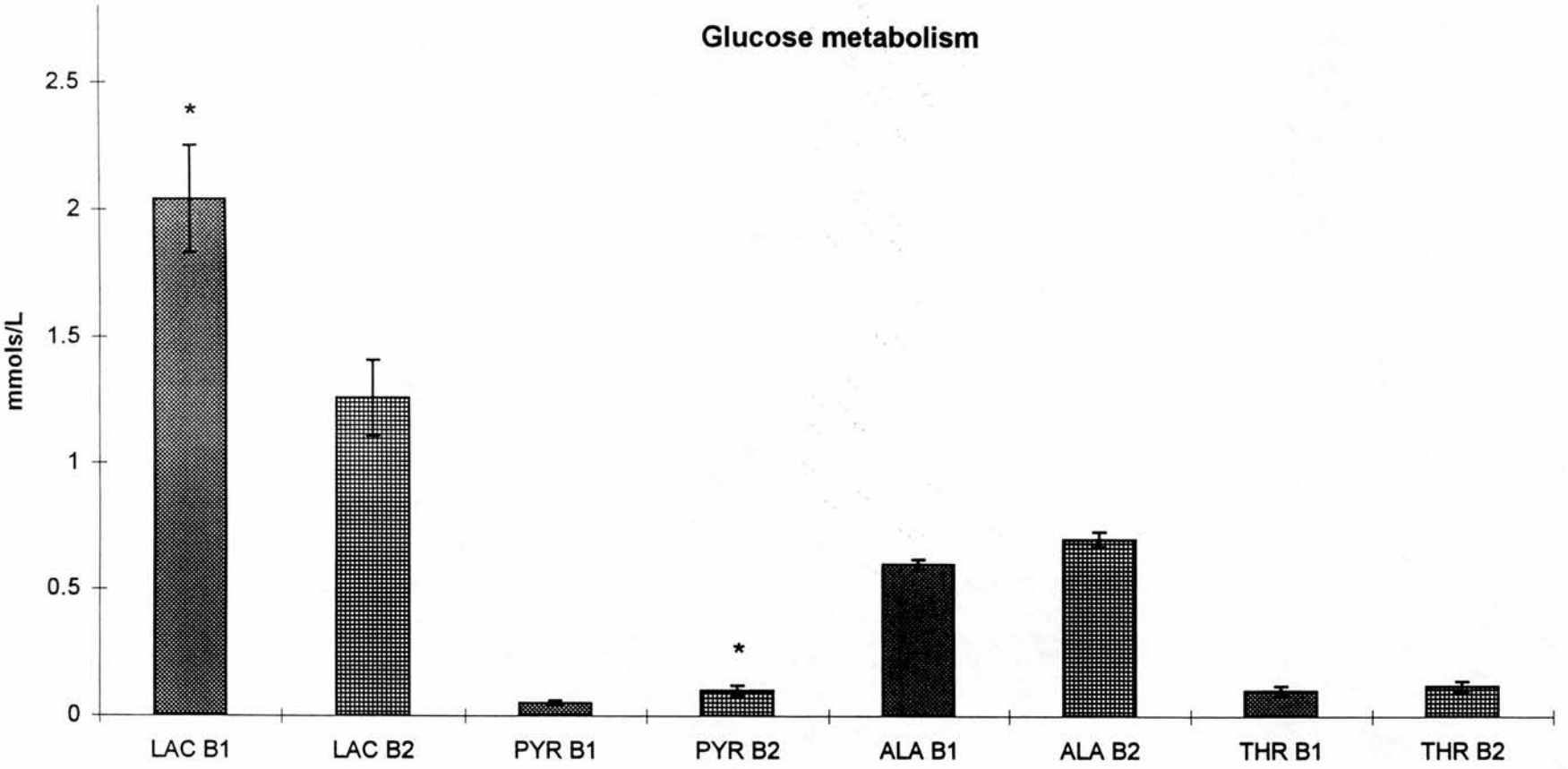
FIGURE 7.17



Concentrations of the intermediates of glucose metabolism in POD FHF on day 1 after admission

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

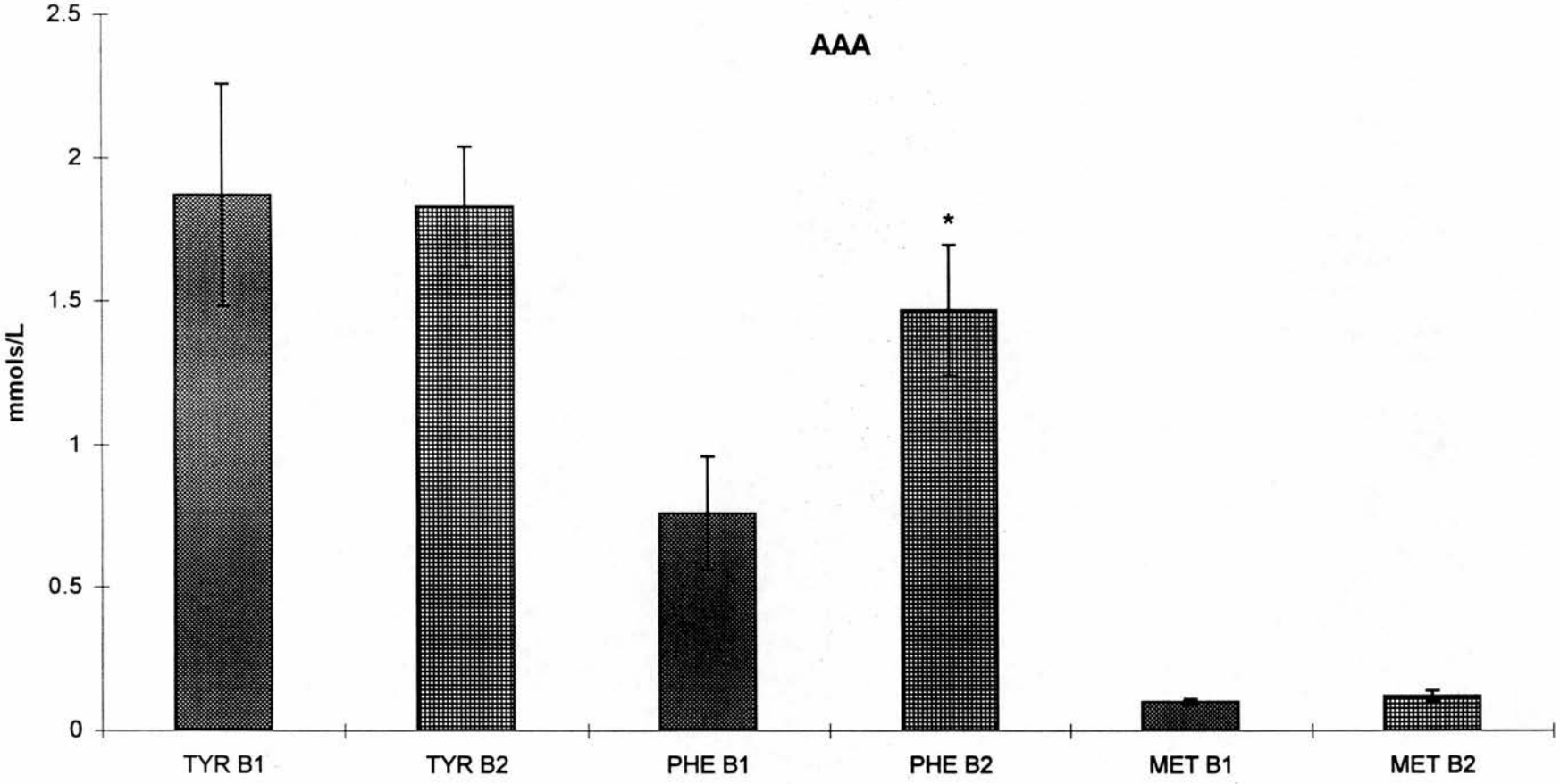
FIGURE 7.18



Concentrations of the intermediates of glucose metabolism in POD FHF on day 3 after admission

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

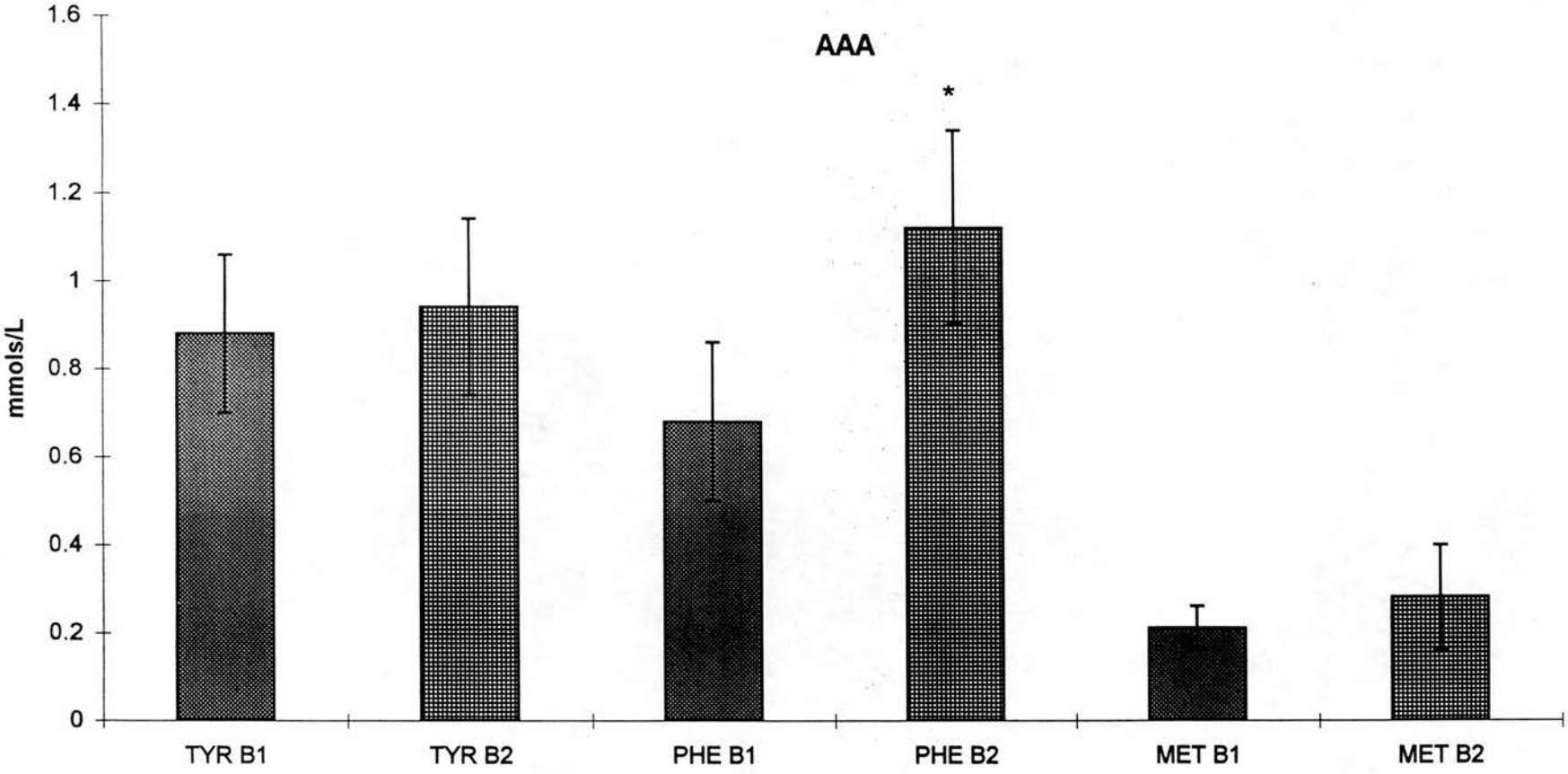
FIGURE 7.19



Concentrations of aromatic amino acids in POD FHf on day 1 after admission

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

FIGURE 7.20



Concentrations of aromatic amino acids in POD FHF on day 3 after admission

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

7.3.4.4 KETOGENESIS

Results concerning the performance of the ketogenic pathway are shown on Figure 7.21 for Day 1 and on Figure 7.22 for Day 3. On Day 1, there were statistically significant differences in the concentrations of ß-hydroxybutyrate ($p<0.042$) and acetate ($p<0.022$). Both were higher in Group B2. There were no differences in the concentrations of acetoacetate. On Day 3, there were statistically significant differences in the concentrations of acetoacetate ($p<0.05$) and acetate ($p<0.044$). Both were higher on Group B2. There were no differences in the concentration of the ketogenic amino acids leucine, isoleucine and tyrosine either on Day 1 or on Day 3.

7.3.4.5 UREA AND GLUTAMINE SYNTHESIS.

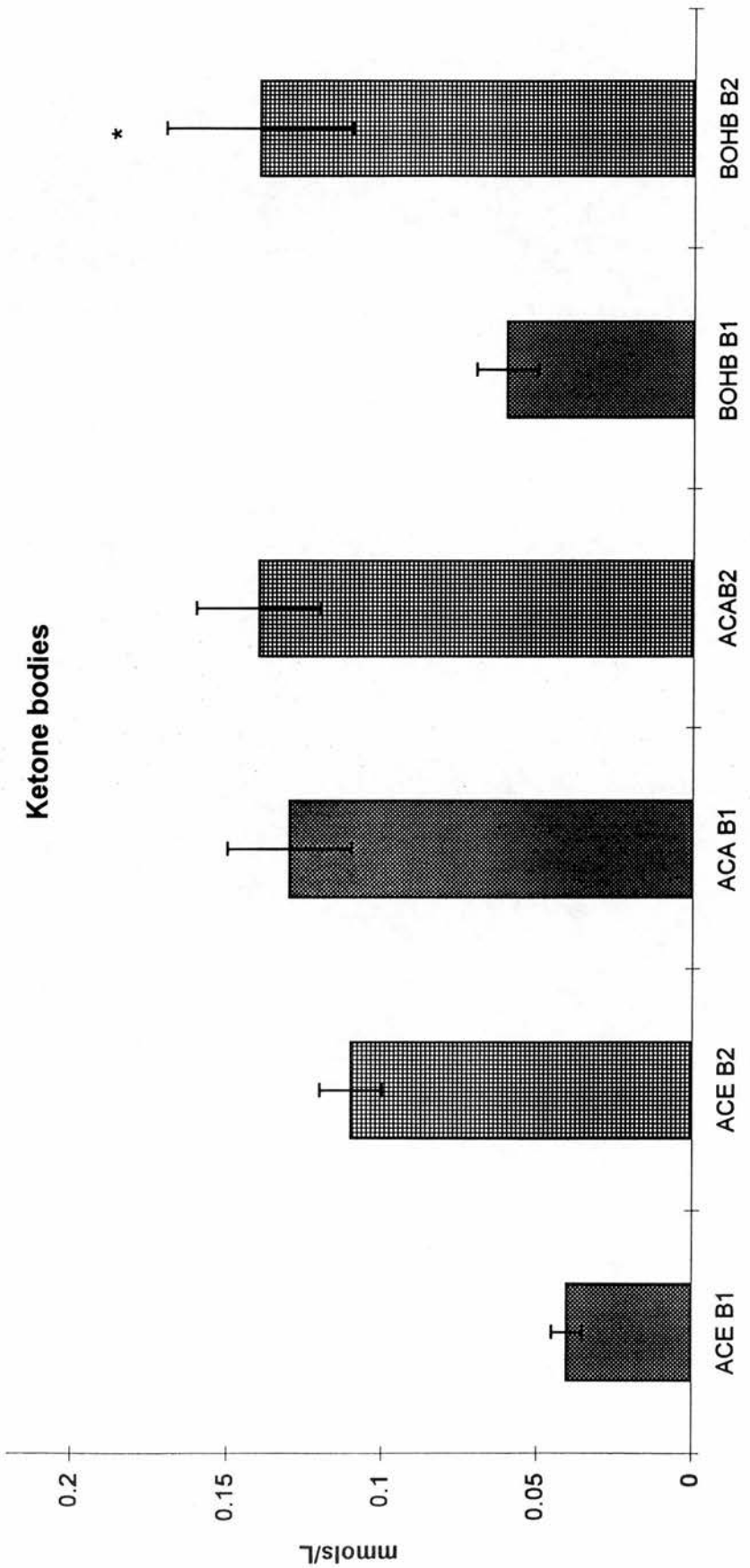
The fate of amines was the same in the two groups. There were no statistically significant differences in the concentration of histidine, glutamate, aspartate, citrulline or arginine, between the two groups on both days. Glutamine concentrations were similar in both groups in both days

7.3.4.6 METHYLAMINES PRODUCTION

We did not find any differences in the production of those substances between the two groups. There were no statistically significant differences in the concentration of Methylamine, Dimethylamine, Trimethylamine and TMAO, on Day 1 and Day 3

No differences were shown in the concentrations of glycerol and myoinositol on both Day 1 and Day 3.

FIGURE 7.21



Concentrations of ketone bodies in POD FHF on day 1 after admission

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

FIGURE 7.22

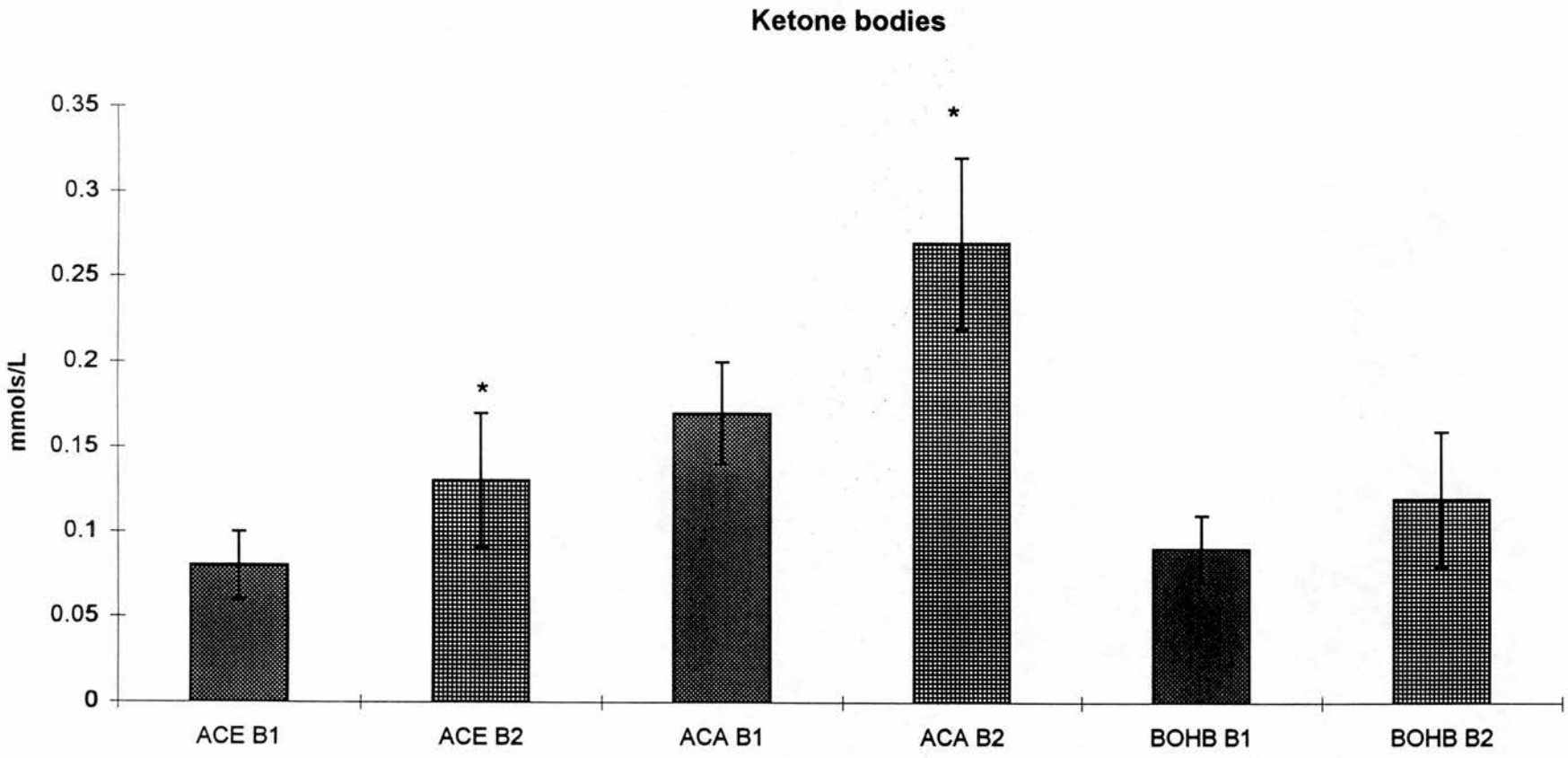


FIGURE 7.22 Concentrations of ketone bodies in POD FHF on day 3 after admission

Results shown are means and the error bars represent SEM. Comparisons are made between the two groups of patients. *p<0.05

7.4 DISCUSSION

Little is known about the metabolic changes that take place in the liver and more specifically intracellularly in that organ during fulminant hepatic failure. We are all aware of the hypoglycaemia, loss of protein synthesis, and increase in lactate production in patients with fulminant hepatic failure.

So far some studies on animal models only, have attempted to elucidate further the abnormalities in key metabolic pathways. Blitzer et al, (1978) in a rabbit model of fulminant hepatic failure, have shown that the concentrations of most amino acids is increased in the first 24 h after injury, compared to controls. In particular they showed that all aromatic amino acids were much increased, methionine was increased, and there was also a slight increase in the concentrations of branch chain amino acids. More specifically there was an increase in the concentrations of leucine with the concentrations of isoleucine and valine remaining almost constant. There was a huge decrease in the concentration of arginine mainly during the second 24 h. There was also an elevation of lactate and creatinine concentrations. Sielaff et al., (1995) in a dog model has shown that there was an increase in the concentrations of proline and alanine from the first 24 h, compared to controls. They also showed that arginine concentration was lower from 12 h onwards compared to controls. They also showed that the overall concentration of aromatic amino acids was elevated and the overall concentration of branch chain amino acids was decreased compared with controls. Other studies in pig and dog models produced similar results (Francavilla et al, 1989; Diaz-Buxo et al, 1997 ; Kalpana et al, 1999).

In this study using 1H NMR Spectroscopy we were able to identify changes in the concentration of amino acids and other metabolic substances in the plasma in

patients with fulminant hepatic failure. In general we were able to identify changes in most amino acids in both the setting of fulminant hepatic failure from paracetamol overdose and in the setting of fulminant hepatic failure from other aetiologies. The spectra of patients looked different from those of controls (Figures 7.23 and 7.24). We are now going to look at metabolic pathways in detail and forward a hypothesis as to why those changes are happening.

Our study confirmed the findings by the animal study by Blitzer et al (1978) in rabbits, showing increased concentrations of the aromatic amino acids, an increase in leucine concentrations, a constant concentration of isoleucine and a decreased concentration of valine. Our results on the aromatic amino acids are in accordance with the study by Sielaff et al (1995), in dogs. As far as we are aware this is the first study to look at an extensive amino acid profile in patients suffering from fulminant hepatic failure

The aromatic amino acids and methionine are primarily metabolised by the liver and their raised concentrations in fulminant hepatic failure are probably due to impaired liver metabolism and portosystemic shunting of blood.

The story is more complex for the branch chain amino acids. The normal liver does not play a major role in the break down of the branch chain amino acids which are mostly catabolised in the skeletal muscle and kidneys. If we look at branch chain amino acids individually we find that their metabolic fate after the initials transamination and decarboxylation can be very different from one to the other. Leucine is a ketogenic amino acid which can be oxidised to acetyl -CoA. This study provides evidence that ketogenesis is impaired in fulminant hepatic failure, as is also the peripheral utilisation of the ketone bodies. Valine can only be gluconeogenic and

enter the Kreb's cycle and provide towards the production of ATP. As acetyl-CoA is in short supply Kreb's cycle can be fuelled from alternative sources such as valine. Isoleucine can be metabolised in both ways thus being either gluconeogenic or ketogenic in the body. Isoleucine may as well be feeding the Kreb's cycle in fulminant hepatic failure but not as successfully as valine.

In the case of glucose metabolism similar changes were observed in both groups of patients. As most patients with fulminant hepatic failure are treated with continuous intravenous infusions of glucose it was impossible to measure accurately the effect of fulminant hepatic injury on glucose concentrations accurately. We then looked at metabolites of glycolysis. There was significant elevation in the concentrations of lactate and pyruvate in patients compared to controls. This is in accordance with observations in humans (Strauss et al, 1997) and animal studies (McMennamy et al, 1965; Blitzer et al, 1978) and shows that although glucose in broken down, there is preferentially anaerobic glycolysis taking place. This is supported also by the extremely low concentration of valine in patients compared to controls, showing that the Kreb's cycle is getting fed by alternative sources to pyruvate. There is an increase in the concentrations of the glucogenic amino acids alanine and glycine. We propose that this is a manifestation of impaired gluconeogenesis and is in accordance with animal studies (Blitzer et al, 1978). Both alanine and glycine can be transformed to pyruvate and enter the glucogenic pathway at that point. There was no change in the concentration of threonine which can be an alanine precursor. We can postulate that this happened because as alanine was not used there was no need for the absorption of threonine. Aspartate concentrations were also elevated in patients compared with controls. Aspartate can be transformed

to phosphoenolpyruvate and enter the glucolytic or neoglucogenic pathway. Elevated concentrations of aspartate support the view that although there is a lot of pyruvate, it is not used appropriately and it is mainly shifted to lactate in an effort for ATP production in an unfavourable environment.

If we look at ketone bodies, which are compounds mainly formed by the liver but exported to other organs for consumption, there was a significant increase in concentrations of acetoaceatate, acetate and 3-hydroxybutyrate if we compare patients with controls. These findings support the hypothesis that the liver early on during fulminant hepatic failure, is able to produce ketones but the target organs are unable to metabolise them. This is supported by evidence that ketogenic amino acids like isoleucine, tyrosine and phenylalanine showed increased concentrations in the plasma of patients. As ketone bodies are not consumed they seem to exerce a sort of negative feedback loop to the their own production process.

We were also able to look at the urea cycle and glutamine synthesis. As urea is undetectable in plasma by ^{1}H NMR Spectroscopy, we have looked at amino acids that feed the cycle and intermediates of the urea cycle. Concentrations of histidine were significantly higher in patients than controls. Histidine is a precursor for glutamate in normal hepatocyte metabolism. Surprisingly, glutamate concentrations were not elevated whereas glutamine concentrations were. We could conclude from this observation, that although there was some production of glutamine, liver and other cells in bad shape due to general critical condition of the patient do not use glutamine to DNA production. Concentrations of citrulline were elevated and so were concentrations of arginine. This was in conflict with the results from animal models(Sielaff et al, 1995, Fahey, 1957) showing that arginine concentrations were

greatly reduced early on. From our results we can conclude that as intermediates from the urea cycle are accumulating not enough urea is produced and the body tries to find other ways to metabolise ammonia. We have evidence that there is an increased production on methylamine and an increase in the concentration of TMAO, which showed that the cells have managed to incorporate ammonia into other substances.

We did not detect any changes in the concentration of glycerol which favours the hypothesis that fatty acid oxidation is not impaired early on in fulminant hepatic failure. Myoinositol concentrations remained unchanged in patients and this could be a sign that changes in osmolytes in fulminant hepatic failure are happening late on in the course of the disease.

In the first part of this chapter we have shown that fulminant hepatic failure produces a vast array of metabolic impairments in patients suffering from it. There were significant changes compared to controls and there was a pattern emerging for anaerobic glycolysis and loss of neoglucogenesis impairment of ketogenesis and possible impairment of urea synthesis.

In the second part of this chapter we looked at differences in metabolic abnormalities comparing between severely and moderately affected patients from both groups and we also looked at progression of abnormalities in time to try and identify possible differences between patients whose aetiology was paracetamol overdose and those whose aetiology was not, during the course of the disease.

In the second part of this chapter we provide some evidence that metabolic changes in the setting of fulminant hepatic failure start quite early in the course of the disease. We also provide evidence to show that aetiology can be a factor determining

how quickly the derangement of the metabolism will settle in. We provide evidence that patients with fulminant hepatic failure seem to lose early on the ability for gluconeogenesis and also are unable to generate energy in the form of ATP by using aerobic metabolism, with a significant accumulation of lactate as a result. Those who suffer fulminant hepatic failure due to paracetamol overdose showed evidence of impaired ketogenesis during the first 24 h post admission. This phenomenon came on later in patients with fulminant hepatic failure from other aetiologies.

Looking at the glycolytic pathway lactate was high from day 1 in both groups. It was significantly higher in patients with severe disease in both groups studied. High lactate was observed in a pig and a dog model of fulminant hepatic failure during the first 12 h (Diaz-Buxo et al, 1997; Kalpana et al, 1999). Concentrations of alanine were mirroring those of lactate. They were elevated from day 1 in both groups. There was also significantly higher alanine concentrations in patients with severe disease in both groups studied. We believe that this reflects an inability of the hepatocytes to proceed to gluconeogensis during the course of fulminant hepatic failure. There were high concentrations of valine in patients with non paracetamol overdose fulminant hepatic failure. We think that this shows that the Kreb's cycle does not feed from glucogenic amino acids. There is an obvious difference in the fate of pyruvate between the two groups. In the non paracetamol overdose fulminant hepatic failure group it was significantly lower in patients with severe disease. This can be linked to the high concentrations of lactate, alanine and valine and shows that although there was a consumption of pyruvate, the cells were unable to further synthesise pyruvate from glucogenetic mechanisms. In the paracetamol overdose fulminant hepatic failure group the concentrations of pyruvate in patients with severe

disease were significantly lower from day 1. This is an obvious change from the other group and we could explain it as follows. Pyruvate is a central player in glucose metabolism and as levels of lactate in those patients are generally higher, we postulate that patients are unable to form new pyruvate through glycolysis, from an early stage but they are still capable of turning it into lactate and acetyl-coA. So, from our results we have shown that although there was impairment of glucose metabolism early on in both groups it was more profoundly affected in patients with severe disease due to paracetamol overdose. By looking at alanine, lactate and pyruvate concentrations on the first day post admission we were able to identify with accuracy, those patients that were going to develop severe liver failure later and this could be a sensitive prognostic criterion.

If we look at ketogenesis and ketone bodies consumption we have found that in severe disease the concentrations of ketone bodies were significantly higher by day 3 in non paracetamol overdose patients. On the contraryin the POD group severely affected patients exhibited lower concentrations of ketone bodies on day 1 and on day 3. In absolute values there was an increase in the concentration of the ketone bodies on day 3 in all POD patients. A possible explanation is the early impairement of gluconeogensis prevents the optimal function of the krebs cycle which needs acetyl-CoA and ketone bodies are able to provide for it. As the situation becomes more severe the Krebs cycle is definiftely impaired and does not use acetyl-CoA any more, thus there is an increase in the concentrations of ketone bodies. We found that the concentrations of some of the ketogenic amino acids was also higher in patients with severe disease. In the non POD group isoleucine concentrations were higher by day 3 and in the POD group concentrations of phenylalanine were higher from day 1. Again

looking at acetate concentrations on the first day post admission we were able to identify with accuracy, those patients that were going to develop severe liver failure later.

There were no differences in urea synthesis or glutamine concentrations according to severity of liver disease. It seems that severity or aetiology did not affect those two metabolic pathways. As citrulline concentrations were higher in patients with severe disease in non POD fulminant hepatic failure this might be an clue that urea synthesis was even more impaired in that setting. In accordance with that hypothesis no differences between severity were observed in the accumulation of methylamines and TMAO .

We did not detect any changes in the concentration of glycerol which favours the hypothesis that fatty acid oxidation is not impaired in fulminant hepatic failure during the first 72 h. Myoinositol concentrations remained unchanged during the time course of the study, in patients regardless of severity and aetiology and this could be a sign that changes in osmolytes in fulminant hepatic failure are happening late on in the course of the disease.

In conclusion this part of the thesis has shown that patients with severe paracetamol overdose tend to have more protracted metabolic disturbances earlier compared to the other groups. As the course of the disease progresses, then patients with severe non paracetamol overdose exhibited similar metabolic abnormalities to those with POD and they were quite similar by 72 h. we have also shown that there are differences between the severely and moderately severely ill patients in both groups and these indices could be used as early prognostic markers in the setting of fulminant hepatic failure. Although this is a small scale study it shows clearly that

metabolic abormalities in patients with liver disease are happenning early in the course of the disease and progression to severe course can be predicted early in the disease.

CHAPTER 8

CHAPTER 8

CRITERIA PREDICTIVE OF OUTCOME IN FULMINANT HEPATIC FAILURE

8.1 INTRODUCTION

In the previous chapter we have shown that there are early biochemical changes manifested in patients with fulminant hepatic failure. Looking at admission bloods we have shown that glucose metabolism is affected quite early in all settings of fulminant hepatic failure. Furthermore, in patients with fulminant hepatic failure caused by paracetamol overdose it was shown that the ketone bodies production and break down were significantly impaired.

The only treatment which has been validated in patients with severe fulminant hepatic failure is orthotopic liver transplantation. The results of orthotopic liver transplantation in that setting have been steadily improving and reported one and five year survival rate at different centres ranges between 60 and 80% (Asher et al 1993; Wei et al, 1997). It is well recognised though that patients transplanted following fulminant hepatic failure have a stormy post -operative time and the one and three month survival is less than for patients transplanted for chronic liver failure. This can be attributed to the poor clinical condition of the patients before the operation but also to the occasional use of ABO-incompatible or inferior quality grafts due to the emergency and shortage of donors (Plevris et al, 1998).

In the UK clinicians apply almost universally the King's prognostic criteria to assess eligibility of patients for otrthotopic liver transplantation. Table 1.4 shows both sets of criteria; one for patients suffering fulminant hepatic failure after a

8.2 MATERIALS AND METHODS

8.2.1 PATIENTS

The same patients, as in Chapter 7 were included in our study. Blood collection was performed as discussed in Chapter 7. Blood was also collected at the same time for measurement of full blood count, coagulation, liver function tests , urea, creatinine and electrolytes and blood gases.

8.2.2 NMR Spectroscopy monitoring

Samples were prepared and quantitation of concentrations of substances were made as discussed in Chapter 7. Data were acquired as discussed in Chapter 7. We measured the concentration of the following substances in plasma using NMR spectroscopy: Acetate, acetoacetate and ß- hydroxybutyrate as they are the main ketone bodies mainly exported by liver cells, glucose, alanine and threonine concentration in the plasma as a measure of glycolysis and gluconeogenesis to monitor the cells energy requirements, lactate and pyruvate as a measure of the active aerobic and anaerobic glycolysis. The concentrations of aromatic amino acids tyrosine and phenylalanine and methionine and the concentrations of branch chain amino acids leucine, isoleucine and valine were measured, as they were shown to be deranged in patients with acute and chronic liver failure and in patients with encephalopathy (Souters et al, 1987; Morgan et al, 1989). The concentrations of glutamate, glutamine, histidine and arginine were measured, as indices of active transamination and urea synthesis. We have also measured concentrations of glycerol

as evidence of on going fatty acid oxidation. Finally we have measured the concentrations of methylamine, dimethylamine, trimethylamine and TMAO as they are thought to be produced by acute injury to hepatocytes. Myoinositol an essential osmotic buffer was measured as well.

8.2.3 Biochemical data .

We have looked at parameters for liver synthetic function and liver necrosis, for renal function and for the coagulation cascade. We have also looked at blood gases results on admission

8.2.4 Statistical analysis.

Separate analysis was performed for fulminant hepatic failure from paracetamol overdoses and the fulminant hepatic failure caused by other aetiologies. To compare between groups we used the Student's t-Test for parameters with non-missing values and the Mann Witney U test for parameters with missing values. Values are expressed as mean (range and Standard error). A p value of<0.05 was taken as statistically significant (two- tail test of significance). To construct our predictive model we used multiple logistic regression analysis with cut off values. We used multiple logistic regression to identify variables that are independent predictors of outcome in our population. We then used stepwise forward logistic regression analysis to create a mathematical equation to predict the outcome of patients with FHF. Using those values we then calculated the specificity, sensitivity, positive and negative predictive value for our predictive model.

8.3 RESULTS

8.3.1 NON POD.

In total 20 patients with non -POD FHF died or were transplanted. As those are censored events for the purpose of analysis they constituted Group A. Fourteen patients survived with maximal supportive therapy and they did not undergo orthotopic liver transplantation. For the purpose of analysis they constituted Group B.

I will just reiterate here the significant results found in the previous study on non POD patients when we looked at amino acids and other key metabolites by NMR on admission bloods. Lactate was significantly higher in Group A ($p<0.023$), pyruvate was significantly lower in Group A ($p<0.014$), alanine was significantly higher ($p<0.032$) and valine was also significantly higher ($p<0.022$) in Group A. No other statistically significant results were obtained.

The analysis of the biochemical data showed that no statistically significant differences between the two groups existed except on two occasions. Bilirubin was significantly higher in group a ($p<0.0053$) and albumin was significantly lower in Group A ($p<0.0041$). There were no differences in prothrombin time between the two groups.

We then performed multiple logistic regression analysis to identify the independent variables to use for our model. We have used the eleven following variables which were either statistically significant or failed to reach significance but there was a trend for difference between the two groups. Lactate, pyruvate, alanine, valine, bilirubin, albumin, prothrombin time, D- dimers, leucine, citrulline and

histidine. After partial correlation and multiple logistic regression analysis the following four variables emerged as the only truly independent variables: Lactate, pyruvate, valine and albumin.

Using stepwise forward logistic regression analysis we were able to create a mathematical model that predicts the outcome of the fulminant hepatic failure on admission bloods. The equation constructed for this model is as follows:

0.2 x (Albumin) - 2 x (Lactate) - 36 x (Valine) - 38 x (Pyruvate)

If the result of this equation is < 2 then, we were able to show that our model had a positive predictive value of 89% , a negative predictive value of 93% , a sensitivity of 94% and a specificity of 86%. We then applied the King's criteria on our population twice once on admission and once overall as is common practice. We found that on admission King' criteria had a positive predictive value of 60 % a negative predictive value of 50%, a sensitivity of 25% and a specificity of 82%. Overall we found that the positive predictive value of the King's criteria was 85%, their negative predictive value was 58%, their specificity was 60% and their sensitivity was 85%

8.3.2 Paracetamol Overdose.

In total 19 patients with POD FHF died or were transplanted .As those are censored events for the purpose of analysis they constituted Group A. Fifteen patients survived with maximal supportive therapy and they did not undergo orthotopic liver transplantation. For the purpose of analysis they constituted Group B.

I will just reiterate here the significant results found in the previous study on POD patients when we looked at amino acids and other key metabolites by NMR on

admission bloods. Lactate was significantly higher in Group a ($p<0.044$), pyruvate was significantly higher in Group A ($p<0.03$)and alanine was significantly higher ($p<0.024$) Acetate was significantly lower ($p<0.022$) in Group A, phenylalanine was significantly lower in Group A ($p<0.011$) and ß-hydroxybutyrate was also significantly lower ($p<0.05$) in group A. No other statistically significant results were obtained.

The analysis of the biochemical data showed that there were statistically significant differences between the two groups. Prothrombin time was significantly higher in group A ($p<0.043$) and albumin was significantly lower in Group A ($p<0.034$). We also observed statistically significant differences between levels of Haemoglobin which was lower in Group A ($p<0.013$), sodium which was higher in Group A ($p<0.035$) and calcium which was also lower in Group A ($p<0.016$).

We then performed multiple logistic regression analysis to identify the independent variables to use for our model. We have used the eleven variables which were statistically significant between the two groups. Lactate, pyruvate, alanine, phenylalanine acetate, ß-hydroxybutyrate, albumin, prothrombin time, haemoglobin, sodium and calcium. After partial correlation and multiple logistic regression analysis, the following seven variables emerged as the only truly independent variables: Phenylalanine, pyruvate, alanine, acetate, sodium, haemoglobin and albumin.

Using stepwise forward logistic regression analysis we were able to create a mathematical model that predicts the outcome of the fulminant hepatic failure on admission bloods. The equation constructed for this model is as follows:

(Haemoglobin) - 400 x (Pyruvate) - 16 x (Phenylalanine)

If the result of this equation is < 93 then, we were able to show that our model had a positive predictive value of 89% , a negative predictive value of 100% , a sensitivity

of 100% and a specificity of 88%. We then applied the King's criteria on our population twice once on admission and once overall as is common practice. We found that on admission King' criteria had a positive predictive value of 85 %, a negative predictive value of 75 % , a sensitivity of 71 % and a specificity of 85%. Overall, we found that the Positive predictive value of the King's criteria was 94 %, their negative predictive value was 93 %, their specificity was 94 % and their sensitivity was 93 %.

8.4 DISCUSSION

In this study we have developed prognostic models that can predict the outcome of patients who suffer from fulminant hepatic failure on admission to the referring hospital. We have studied the paracetamol overdose FHF separately as those patients have a higher incidence of renal failure and metabolic acidosis (O'Grady et al, 1989). Paracetamol overdose fulminant hepatic failure accounts for 80% of admissions to our unit. As this study was set out to construct models to predict outcome in fulminant hepatic failure we decided that the two groups (POD-FHF and non-POD-FHF) should have similar numbers. Forty two patients were admitted to SLTU suffering from FHF from aetiologies other than paracetamol overdose. We managed to collect plasma and study 34 of them. To keep the numbers of patient groups even we also studied the thirty four paracetamol overdoses that fulfilled the fulminant hepatic failure criteria which were admitted following our thirty four non-POD-FHFs.

Our study provides a new model for evaluating patients with FHF. It is based in our previous study which showed that key metabolic pathways in patients with fulminant hepatic failure are impaired as early as 24 hours after admission to the

tertiary referral centre. We have been fortunate to be able to use ^{1}H NMR Spectroscopy to identify metabolic abnormalities in patients with fulminant hepatic failure, by looking at the patients plasma. Our model uses biochemical and dynamic variables and does not take into account static and therapeutic variables such as age, sex, aetiology, duration of symptoms and grade of encephalopathy. This might represent a drawback but the model was developed to show that by using only biochemical parameters it is possible to formulate a credible predictive model.

We have added the most common biochemical indices to our NMR data to try and make our model more accurate. Our result were surprising as the most commonly used biochemical markers either did not show any statistical differences between severe and moderate disease or they were not selected as independent variables by our model. This comes as a surprise as prothrombin time was always thought to be on of the best predictors of outcome in FHF (O'Grady et al, 1989; Harrison et al, 1990; Takahashi et al, 1994; Anand et al, 1997). One possible explanation for our results is that we have looked at indices on admission to our unit and it is well known that prothrombin time is a good marker but probably two or three days after admission and its values are changing rapidly. The same reasoning would apply to creatinine as renal failure in POD is a rather late onset. Bilirubin in the non-POD-FHF situation was an independent variable but our final model excluded bilirubin as there were other more powerful predictors.

If we look at our non-POD-FHF model the variables included were lactate, pyruvate, valine and albumin. Admission lactate is known to be a predictor of outcome in fulminant hepatic failure. (Bernal et al., 1999). It comes as no surprise as we have shown that anaerobic glycolysis is the norm in fulminant hepatic failure.

Pyruvate is a central metabolite in the glycolysis and gluconeogenesis pathway. Its lower values in the non-POD-FHF suggest less production as gluconeogenesis is impaired and also that more pyruvate is shifted to lactate to meet the energy requirements of the cells. The higher concentrations of valine in severely affected patients may suggest that already on admission the disease has affected adversely the ability of the muscle to metabolise branch chain amino acids. In other words, already on admission we are faced with a multi-organ disease, that has disabled the basic life sustaining metabolic pathways of energy production. We do not have an explanation for the significance and the inclusion of low albumin, other than it may represent a previous general condition of the patient which was not entirely healthy.

If we look at our POD-FHF model the striking observation is that the most powerful predictor seem to be admission haemoglobin with a cut-off value of 100g/l. This is of course an abnormally low level for both sexes. We can offer no explanation for this finding and we can only look at it as a strange finding that we will try and clarify in a future study. In contrast to the non-POD-FHF situation we have observed a significantly higher pyruvate level in severely affected individuals. As lactate in our study group was significantly elevated in severely affected patients we can hypothesise that gluconeogenesis is impaired at this point in POD-FHF and more production of pyruvate is still taking place. However at this point glucose consumption has inevitably lactate as an end product thus, there is accumulation of lactate. We have found lower levels of phenylalanine in severely affected POD patients and this may simply pepresent an early surrogate marker pof severe encephalopathy in the fulminannt setting. .

Our study tried to identify prognostic markers of outcome in patients with FHF on admission bloods. To our knowledge only one previous study has accomplished that and they looked at patients with FHF due to viral hepatitis (Bernuau et al, 1991). The currently used King's college criteria, when applied to our patients, confirmed that they represent a valid set of criteria if applied overall and if parameters are measured every day. They were less effective in predicting outcome on admission. It is well recognised that in FHF time is very precious and often patients on the waiting list die as a donor organ never becomes available. The fact that our criteria may be able to predict outcome on admission could offer those patients a time window that could be life saving as they could be placed on a super-urgent waiting list earlier.

We have used NMR Spectroscopy for the measurement of many of our variables mainly the amino acids. Although this technique is not familiar to the medical world, extensive work has proven that it can be used to measure concentrations of low molecular weight substances in biofluids. Facilities exist in all major University centres in the UK and even centralised facilities for our purpose could be envisaged.

In conclusion our study enabled us to construct two models to predict outcome by measuring concentrations of markers on admission plasma, with a high sensitivity and specificity. Further work is underway to validate these models prospectively.

CHAPTER 9

METABOLIC ABNORMALITIES IN PATIENTS WITH CHRONIC LIVER DISEASE AND CHRONIC HEPATIC ENCEPHALOPATHY

9.1 INTRODUCTION

Most chronic liver injuries would result in cirrhosis, which is a combination of hepatic fibrosis and nodular regenerative hyperplasia. Amongst the many causes of chronic liver injury, a distinct few account for the majority of cases. Alcohol is the most common cause in the developed world and Hepatitis B infection in Asia and Africa. Currently world wide, the proportion of cirrhosis due to chronic Hepatitis C infection is on the increase (Plevris et al, 1998).

One of the major complications of cirrhosis is chronic hepatic encephalopathy. This is a complex neuropsychiatric syndrome which has a potential for full reversibility. It is characterised by global depression of the central nervous system and has different degrees of severity.(West Haven classification, (Figure 1.3)). The syndrome is usually episodic and relapsing but some patients exhibit a chronic protracted course (Jalan et al, 1996).

Cirrhosis is associated with alterations in proteins and amino acids metabolism, including diminished urea formation (Du Ruisseau et al, 1956) and hyperammonaemia (Fahey, 1957). Most studies that have looked at amino acid metabolism in cirrhosis would agree that there seems to be a recognisable pattern in the plasma amino acid profile with an elevation of aromatic amino acids and methionine and reduced levels of branch chain amino acids (Morgan et al, 1982;

Morgan, 1990; Plauth et al, 1990). Although this pattern has been used as the basis for the false neurotransmitter theory in the pathogenesis of chronic hepatic encephalopathy (Fischer et al, 1971) not many studies have proved that the pattern observed in cirrhosis is valid for encephalopathy too (Fischer et al, 1978; Morgan et al, 1982). Furthermore, it is now well accepted that encephalopathy due to acute hepatic failure is a different entity and the mechanisms contributing to the CNS dysfunction in the two diseases might be different.

Most studies have not concentrated in patients with chronic encephalopathy only. The one study that has, focused on disproving that the BCAA to AAA ratio is a valid predictor of encephalopathy grading (Morgan et al, 1978).

Other biochemical abnormalities are believed to be present in cirrhotics. Gluconeogensis is impaired and hyperammonaemia and diminished urea production makes it necessary for the body to find other pathways for nitrogen elimination (Mc Cullough et al, 1993).

In this study we have studied amino acid patterns in cirrhosis, ketone bodies, intermediates of glucose metabolism and urea cycle intermediates and nitrogen by products. For this purpose we have used ^{1}H Nuclear Magnetic Resonance Spectroscopy which is an easy method for measuring concentrations of low molecular weight substances in low concentrations in complex biological fluids without destroying the original samples.

The aim of our study was to investigate amino acid patterns and protein metabolism, ketogenesis and nitrogen detoxification in patients with cirrhosis and to compare those findings with patients with cirrhosis during an episode of hepatic encephalopathy.

9.2 Materials and Methods

9.2.1 Patients.

We studied patients with stable cirrhosis (group A, 18 patients), patients with stable cirrhosis during an episode of hepatic encephalopathy (group B, 18 patients) and normal controls(group C, 17 subjects), the same group we used as controls in Chapter 7. Individual characteristics of the patients in the two groups are shown in Table 9.1.

Blood was collected from all three groups two to three hours following a main meal as differences in the concentration of amino acids between sexes are less pronounced post prandially, in lithium heparin tubes. It was immediately centrifuged at 2000 g for 15 minutes at 4^0C. The supernatant was then pippetted and aliquoted in 2.5 ml vials. The vials were then stored at -40^0C until NMR analysis.

9.2.2 NMR Spectroscopy

Sample preparation for NMR spectroscopy was performed as discussed in Chapter 7. The same experimental design was used as in Chapter 7 both to acquire spectra from the plasma of the subjects and also to quantitate the concentrations of the observed substances. We have measured the concentrations of the same substances as previously described in Chapter 8.

9.2.3 Statistical analysis

To compare between the three groups we used the three way ANOVA test. Where the ANOVA test was statistically significant the Tuckey test was performed to compare between groups. Values are expressed as mean (range and Standard error). A p value of$\leq$0.05 was taken as statistically significant (two- tail test of significance).

TABLE 9.1

Individual characteristics from patients with cirrhosis and patients with cirrhosis and chronic hepatic encephalopathy (CHE)

	CIRRHOSIS	CHE
AGE	54.3 ± 8.8 yrs	56.8 ± 6.0 yrs
MALES	9	12
FEMALES	9	6
CHILD'S SCORE	7.8 ± 1.6	9.9 ± 2.1
PUGH CLASS A	1	1
PUGH CLASS B	11	6
PUGH CLASS C	6	11

9.3 RESULTS.

Comparisons between the substances observed were made between cirrhotics, encephalopathic cirrhotics and controls following the three way ANOVA model.

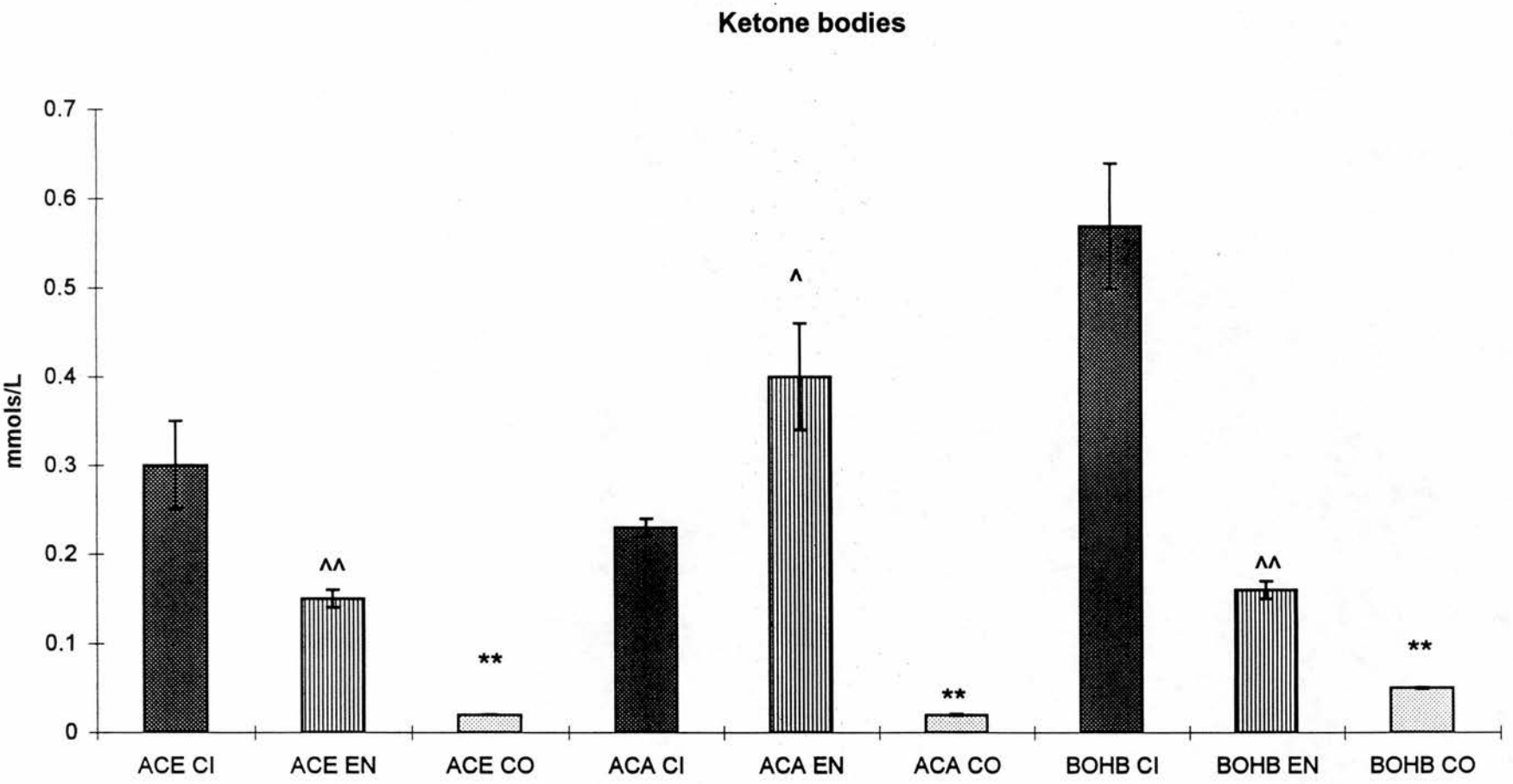
9.3.1 Ketogenesis

Results are summarised in Figure 9.1. We have observed statistically significant differences by three way ANOVA between controls and patients in all three ketone bodies measured. Acetate, acetoacetate and ß-hydroxybutyrate, were all significantly higher in patients than controls ($p<0.01$ in all cases). If we compared between patients with and without encephalopathy, acetate was significantly lower in encephalopathics ($p<0.01$) as was ß-hydroxybutyrate ($p<0.0002$). In contrast acetoacetate was significantly higher in encephalopathics ($p<0.05$).

9.3.2 Aromatic amino acids

Results are summarised in Figure 9.2. We have observed statistically significant differences by three way ANOVA between controls and patients in the two aromatic amino acids and methionine measured. Tyrosine, phenylalanine and methionine concentrations were significantly higher in patients than controls ($p<0.01$ in all cases). If we compared between encephalopathic and non-encephalopathic patients, there were no differences in aromatic amino acids or methionine concentrations.

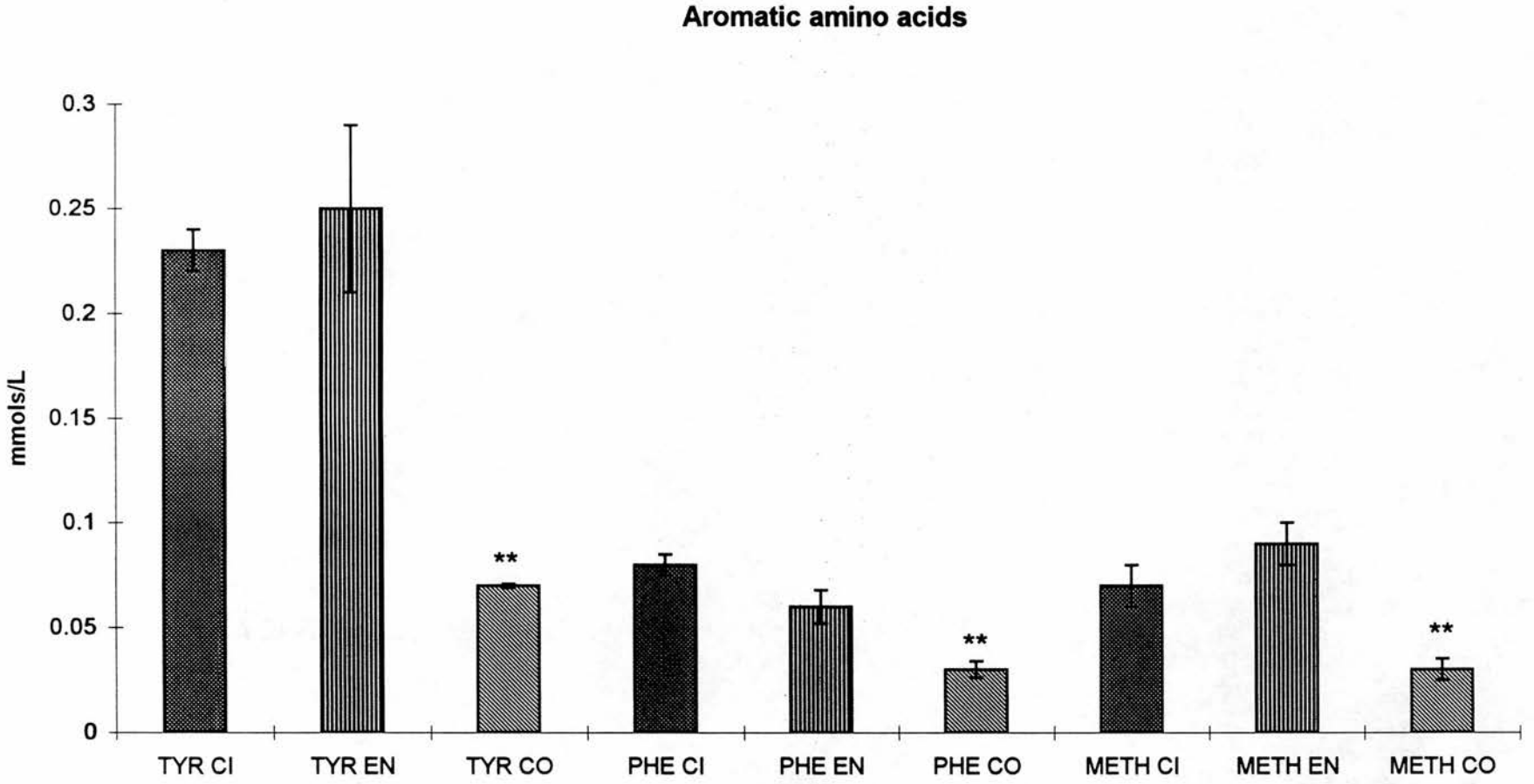
Figure 9.1



Concentrations of ketone bodies in plasma of patients with cirrhosis (CI), Cirrhosis and chronic hepatic encephalopathy (EN) and controls (CO).

Results shown are means and the error bars represent SEM. Comparisons are made between the control group and the two patients groups and between patient groups *p<0.05 ** p<0.01 between controls and patients , ^p<0.05, ^^p<0.01 between patient groups

Figure 9.2



Concentrations of aromatic amino acids in plasma of patients with cirrhosis (CI), Cirrhosis and chronic hepatic encephalopathy (EN) and controls (CO).

Results shown are means and the error bars represent SEM. Comparisons are made between the control group and the two patients groups and between patient groups *p<0.05 ** p<0.01 between controls and patients

9.3.3 Branch chain amino acids

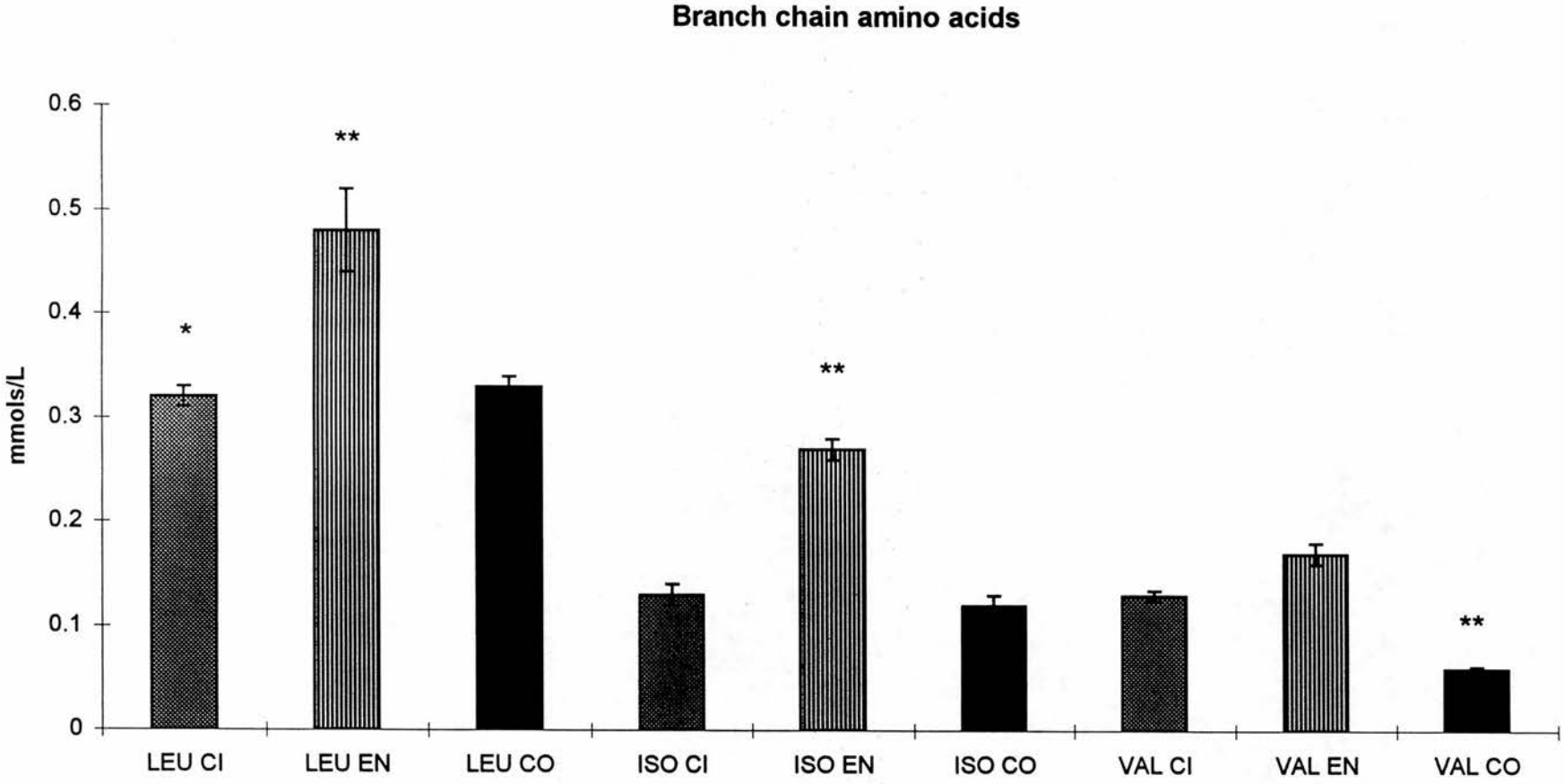
Results are summarised in Figure 9.3. We have observed statistically significant differences by three way ANOVA between controls and patients in all three branch chain amino acids measured. Valine was significantly lower in patients than controls ($p<0.01$ in both cases). Leucine was significantly lower if we compared cirrhotics with controls ($p<0.05$) and higher if we compared encephalopathics with controls ($p<0.01$). Isoleucine was significantly lower if we compared encephalopathics with controls ($p<0.01$) but there was no difference between cirrhotics and controls. If we compare encephalopathic with non- encephalopathic patients, there were no differences in the concentrations of valine. The concentration of leucine was significantly higher in encephalopathics ($p<0.005$) as was the concentration of isoleucine ($p<0.0004$).

9.3.4 Glycolysis and gluconeogenesis.

Results are summarised in Figure 9.4 for lactate and pyruvate. We have observed statistically significant differences by three way ANOVA between controls and patients in both lactate and pyruvate. Lactate and pyruvate concentrations were significantly higher in patients than controls ($p<0.01$ in all cases). There were no differences present if we compared the two patients groups.

Figure 9.5 summarises the results for the gluconeogenesis. We have observed statistically significant differences by three way ANOVA between controls and patients in all four amino acids measured. Alanine, threonine, glycine and aspartate concentrations were significantly higher in patients than controls ($p<0.01$ in all cases). There were no differences present if we compared the two patients groups.

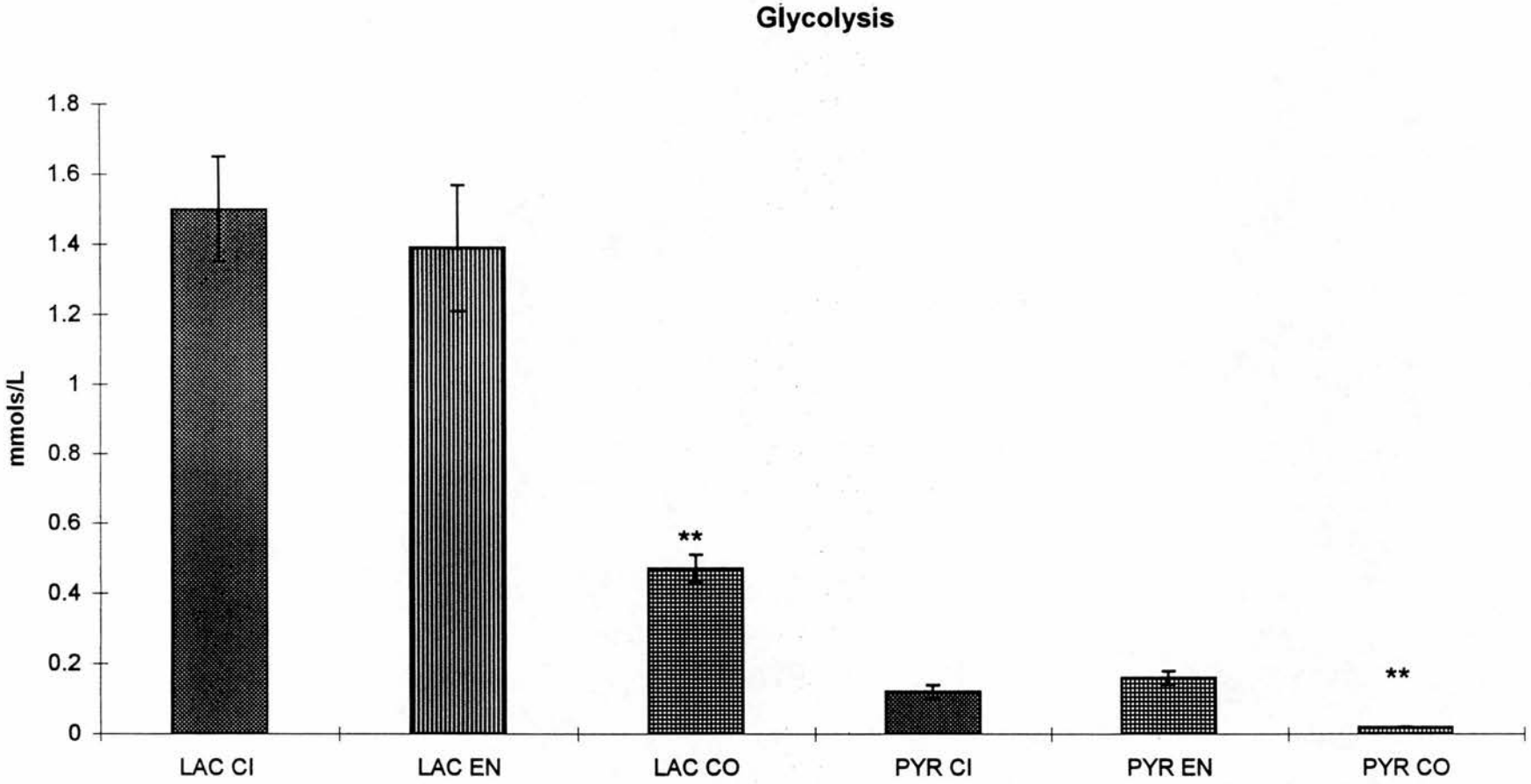
Figure 9.3



Concentrations of branch chain amino acids in plasma of patients with cirrhosis (CI), Cirrhosis and chronic hepatic encephalopathy (EN) and controls (CO).

Results shown are means and the error bars represent SEM. Comparisons are made between the control group and the two patients groups and between patient groups *p<0.05 ** p<0.01 between controls and patients.

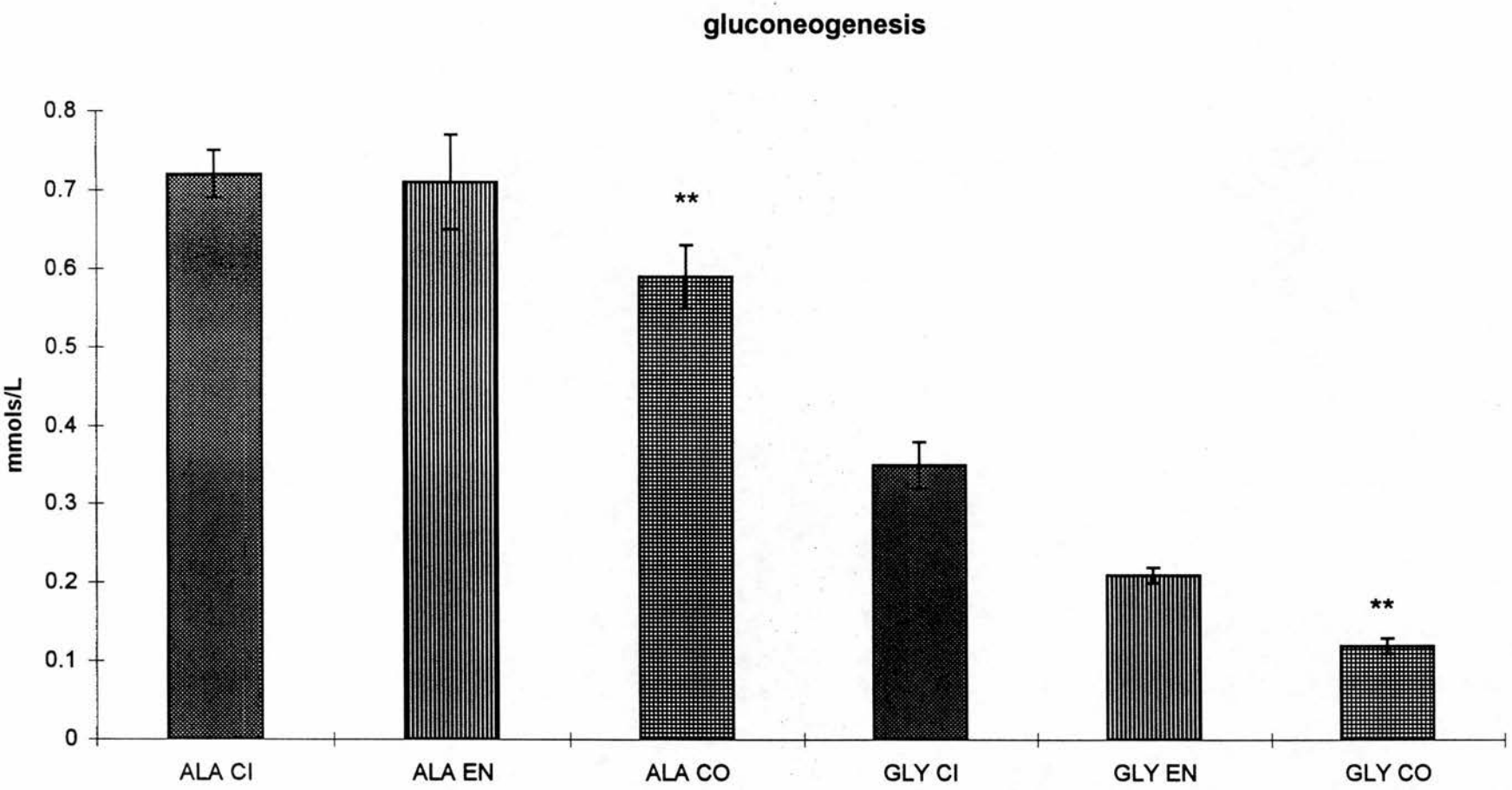
Figure 9.4



Concentrations of glucose metabolites in plasma of patients with cirrhosis (CI), Cirrhosis and chronic hepatic encephalopathy (EN) and controls (CO).

Results shown are means and the error bars represent SEM. Comparisons are made between the control group and the two patients groups and between patient groups *p<0.05 ** p<0.01 between controls and patients .

Figure 9.5



Concentrations of glucogenic amino acids in plasma of patients with cirrhosis (CI), Cirrhosis and chronic hepatic encephalopathy (EN) and controls (CO).

Results shown are means and the error bars represent SEM. Comparisons are made between the control group and the two patients groups and between patient groups *p<0.05 ** p<0.01 between controls and patients .

9.3.5 Urea metabolism and glutamine synthesis

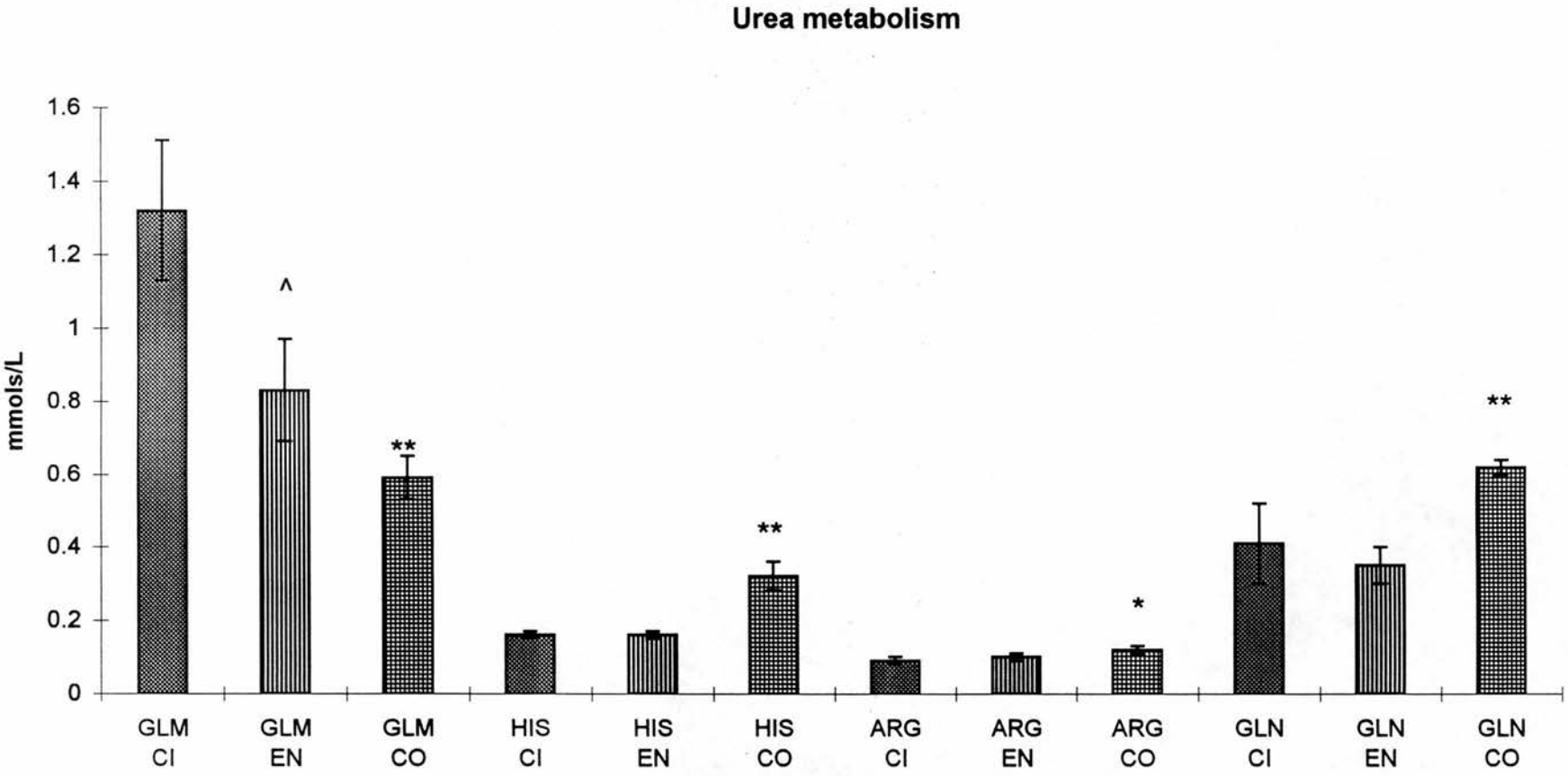
Figure 9.6 summarises the results for the amino acids involved in the urea and glutamine synthesis. We have observed statistically significant differences by three way ANOVA between controls and patients in all four amino acids measured. Glutamine, histidine and arginine concentrations were significantly lower in patients than controls ($p<0.01$ in all cases). Glutamate ewas significantly higher in patients compared to controls ($p<0.01$ in all cases). If we compared between the two patients groups, glutamine concentrations were lower in encephalopathic patients ($p<0.013$). Glutamate, histidine and arginine concentrations were not significantly different

Figure 9.7 summarises the results for the methylamines. We have observed statistically significant differences by three way ANOVA between controls and patients in all three substances measured. Methylamine, dimethylamine and TMAO concentrations were significantly higher in patients than controls ($p<0.01$ in all cases). If we compared between the two patients groups there were no statistically significant differences in the concentrations of methylamine, dimethylamine, and TMAO.

9.3.6 Glycerol and myoinositol.

Figure 9.8 summarises the results for those two osmotically active substances. Glycerol concentrations were significantly higher in patients than controls ($p<0.01$ in both cases). Myo-inositol was significantly higher in cirrhotics ($p<0.015$) but there were no differences between encephalopathic patients and controls. If we compare between the two patients' groups, glycerol concentration was very significantly lower in encephalopathics ($p<0.000$). Myoinositol concentrations were also significantly lower in encephalopathic patients ($p<0.01$).

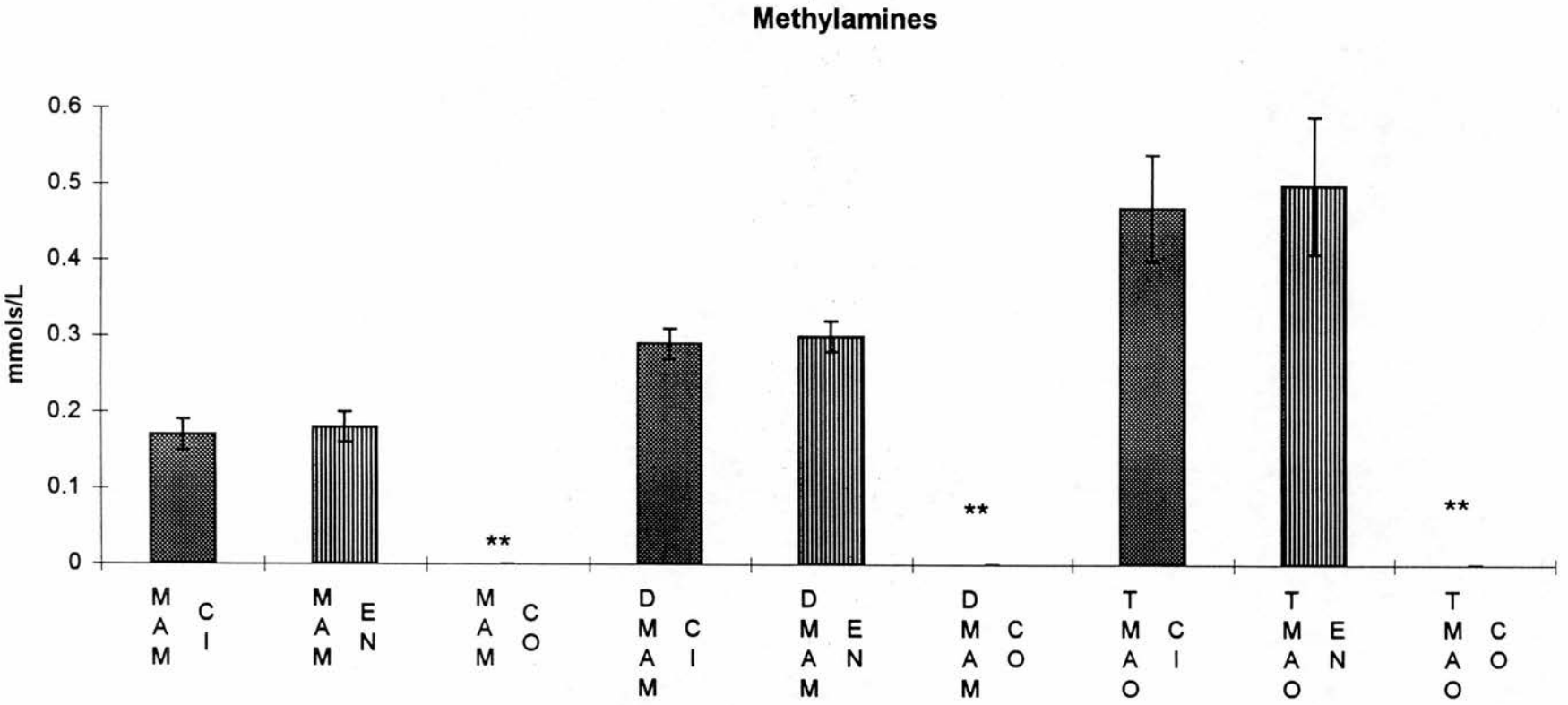
Figure 9.6



Concentrations of urea and glutamine synthesis intermediates in plasma of patients with cirrhosis (CI), cirrhosis and chronic hepatic encephalopathy (EN) and controls (CO).

Results shown are means and the error bars represent SEM. Comparisons are made between the control group and the two patients groups and between patient groups *p<0.05 ** p<0.01 between controls and patients, ^p<0.05, ^^p<0.01 between patient groups

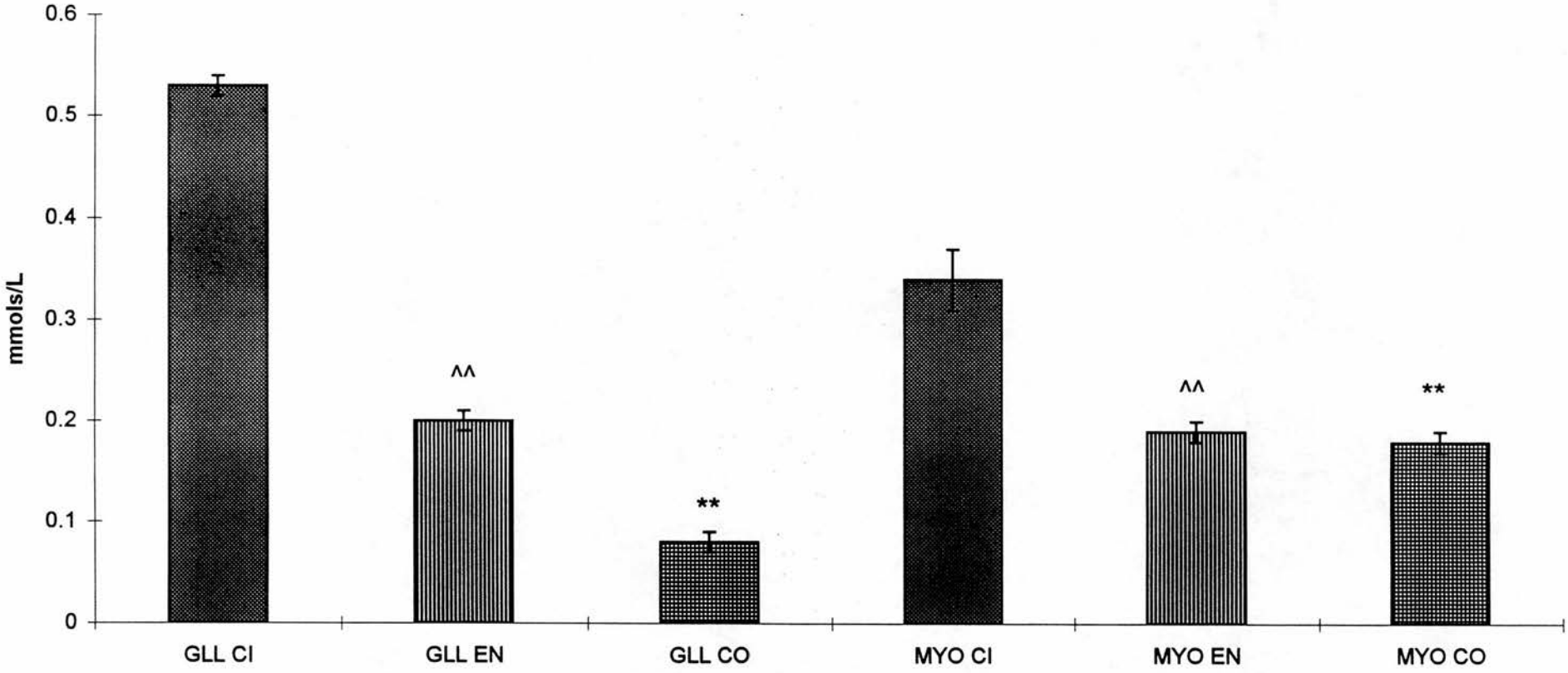
Figure 9.7



Concentrations of methylamines in plasma of patients with cirrhosis (CI), Cirrhosis and chronic hepatic encephalopathy (EN) and controls (CO).

Results shown are means and the error bars represent SEM. Comparisons are made between the control group and the two patients groups and between patient groups *p<0.05 ** p<0.01 between controls and patients ,

Figure 9.8



Concentrations of glycerol and myo-inositol in plasma of patients with cirrhosis (CI), cirrhosis and chronic hepatic encephalopathy (EN) and controls (C0).

Results shown are means and the error bars represent SEM. Comparisons are made between the control group and the two patients groups and between patient groups *p<0.05 ** p<0.01 between controls and patients , ^p<0.01, ^^p<0.01 between patient groups

9.3.7 EDI-2

We should mention here that all spectra from encephalopathic patients exhibited a novel substance (Figure 9.10 and Figure 9.11) which was not present in either the cirrhotics (Figure 9.11) or the controls (Figure 7.24). This substance was present in high concentrations compared to most amino acids (185 -400 μmols/L) .

The ^{1}H NMR spectrum of this substance which we called EDI-2 exhibited four broad peaks at 1.56 ppm, at 3.86 ppm, at 4.16 ppm and at 4.53 ppm. By examining this spectrum, we have hypothesised that EDI-2 is a small molecule that is loosely bound to a big protein molecule such as albumin which forces it to tumble slowly in the solution.

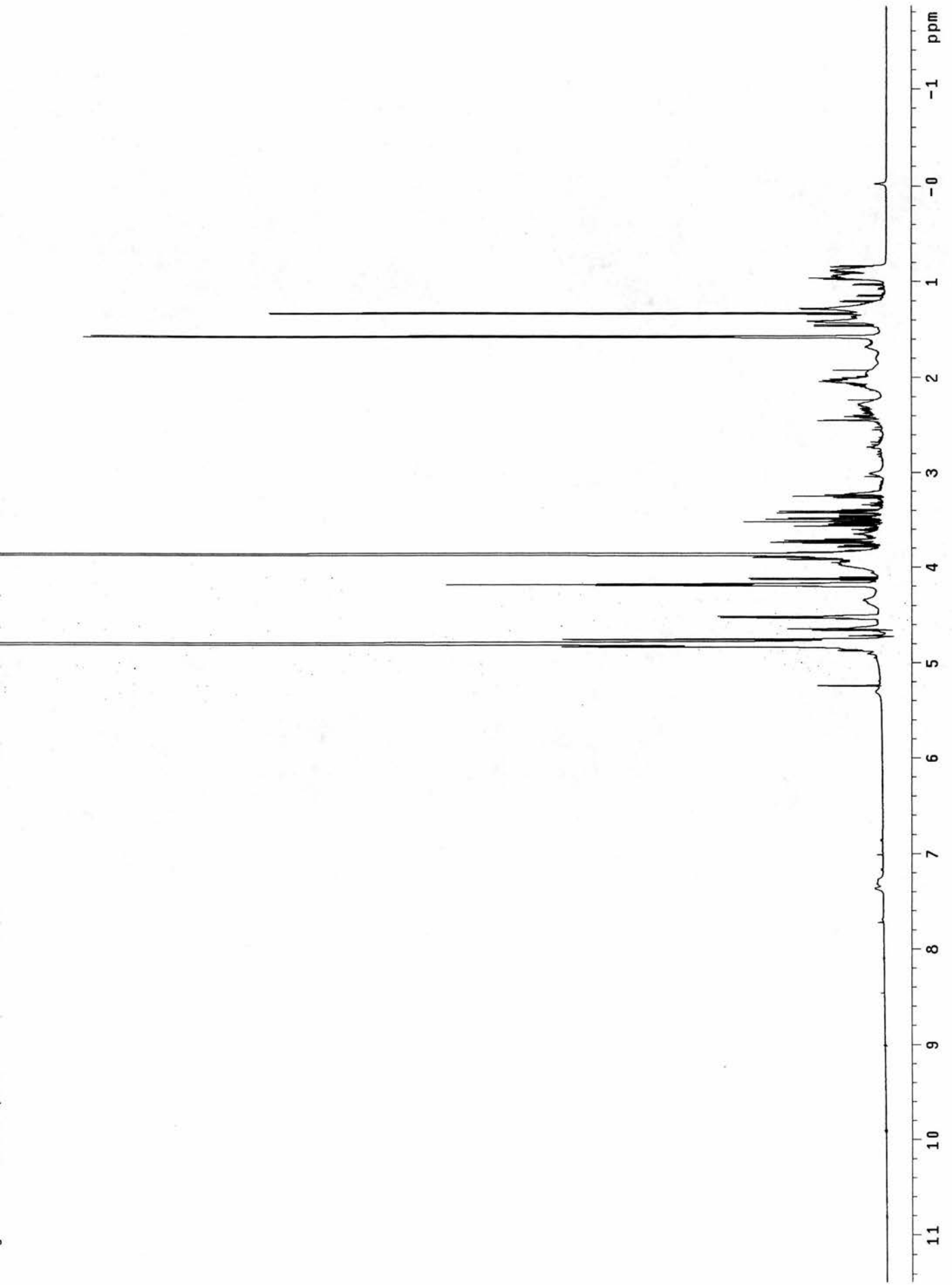
Having excluded the possibility that EDI-2 is a drug, a dye, an excipient or simply an impurity we concentrated on identifying the molecular structure of EDI-2. To find out about the possible similarities of the spectrum of EDI-2 with the spectra of other known substances, we examined reference compounds libraries but no spectrum could be matched with the one from EDI-2. The only spectrum that looked similar was the spectrum of melatonin.

Melatonin is a hormone that is secreted by the pineal gland and the gut and induces sleep. The effects of melatonin on the human body resemble the symptoms of hepatic encephalopathy. It is as if patients with hepatic encephalopathy have an excessive and inappropriate secretion of melatonin.

As EDI-2 was not melatonin, we postulated that it might be one of melatonin's precursors or by-products. To investigate this we used High Performance Liquid Chromatography (HPLC) to purify EDI-2 from the plasma. We then

compared the peaks from the eluent of the chromatography column, with results from HPLC of precursors and by-products of melatonin. We were unable to confirm similarities between EDI-2 and any of the examined substances.

A new round of investigations trying to match EDI-2 and oxindole and its by-products are currently underway as part of our attempt to determine the potential role of EDI-2 in the pathogenesis of chronic hepatic encepaholpathy.


ppm
-1
-0
1
2
3
4
5
6
7
8
9
10
11

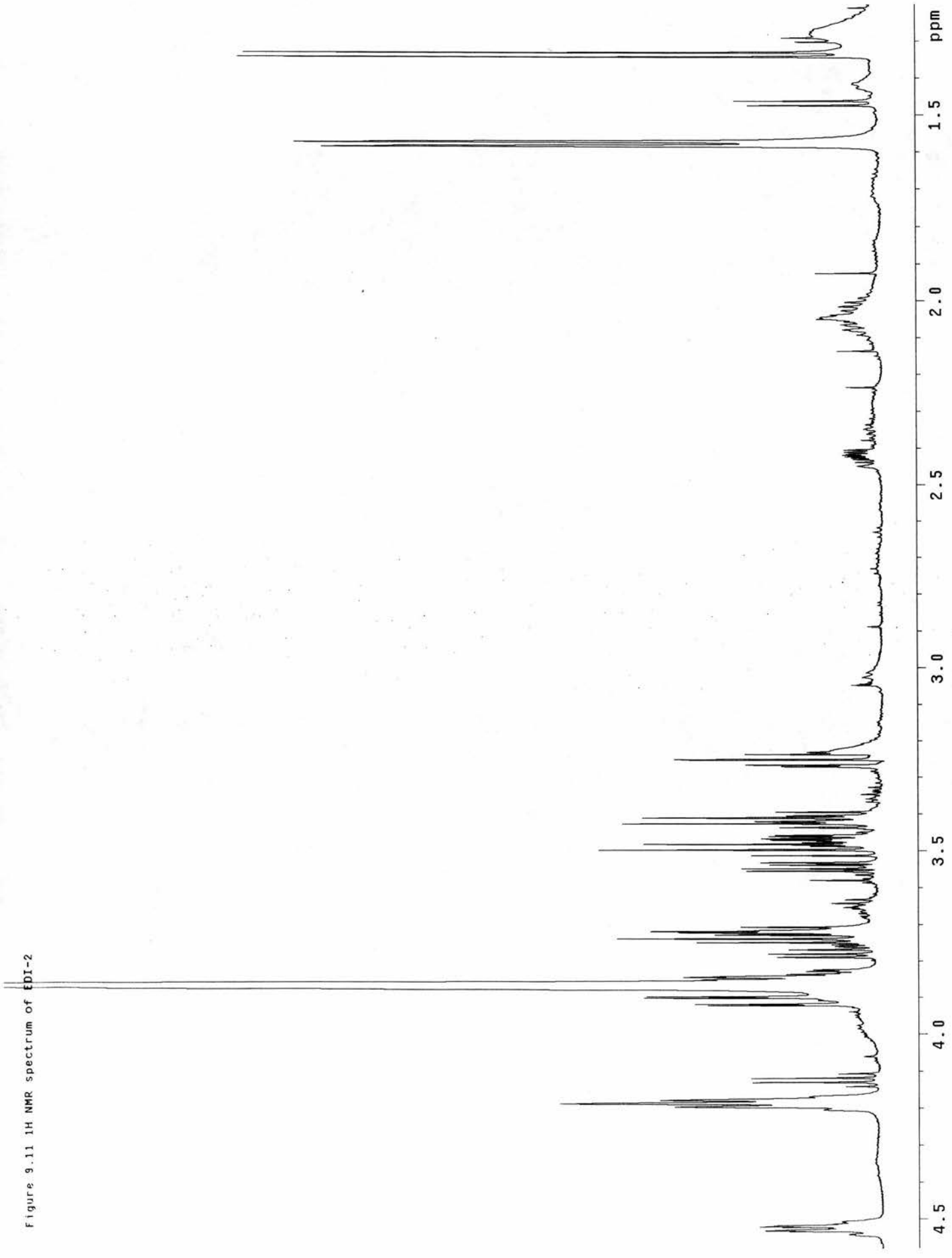


Figure 9.11 1H NMR spectrum of EDI-2

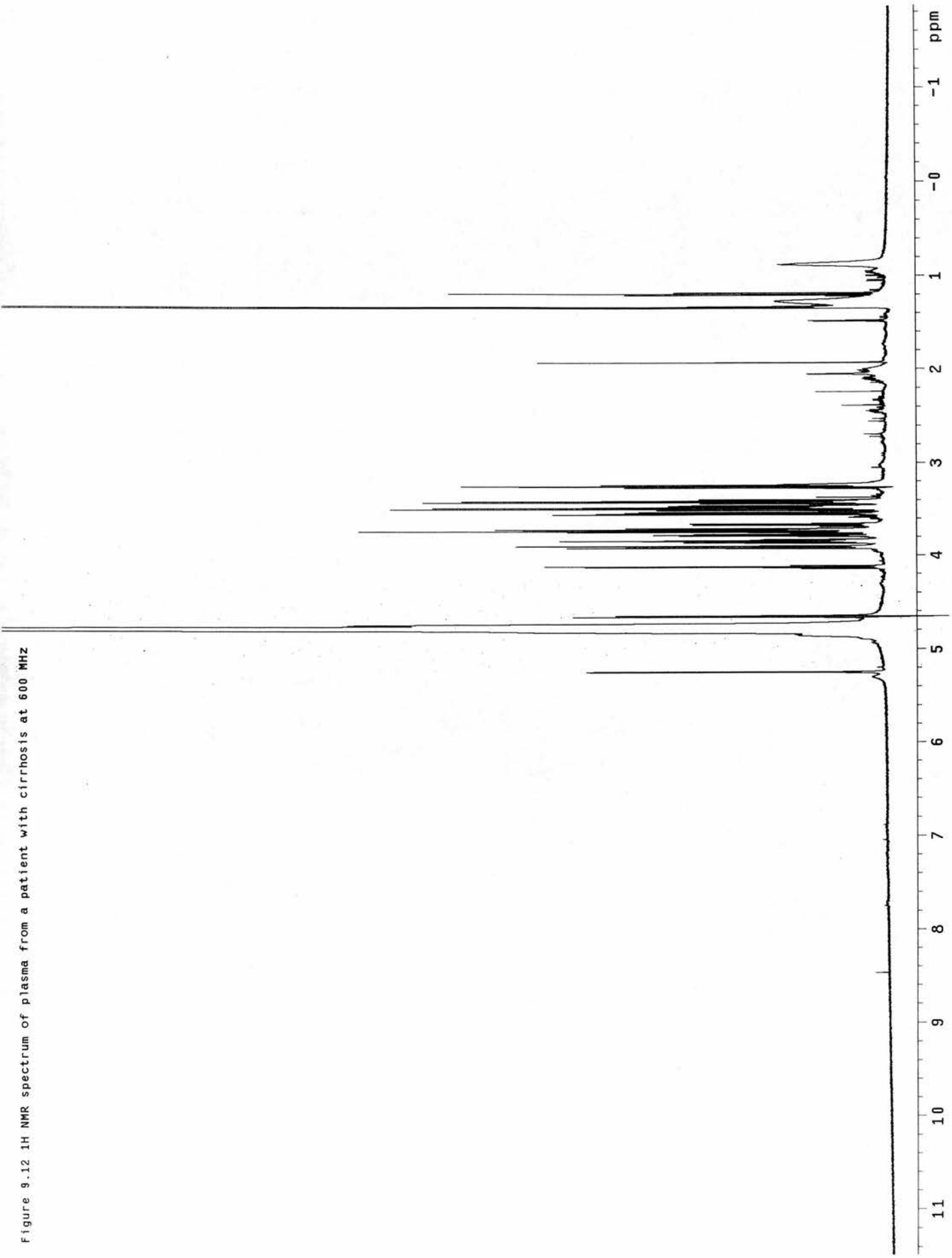


Figure 9.12 1H NMR spectrum of plasma from a patient with cirrhosis at 600 MHz

9.4 DISCUSSION

Our study has confirmed that significant changes occur in plasma concentrations of amino acids and other key metabolites in patients with cirrhosis in the presence or not of hepatic encephalopathy. We will now look at some particular substances in more detail.

Lactate and the amino acids alanine, threonine, glycine and aspartate are major precursors for gluconeogenesis. Pyruvate is also a central substance in glucose metabolism. In both cirrhotics and encephalopathics we found that the concentrations of those substances were uniformly increased. It appears then that gluconeogenesis is generally impaired in cirrhosis and encephalopathy. This would be in accordance with previous studies, in experimental models (Seglen 1976; De Blaauw et al, 1997).

Our study showed that the concentrations of ketone bodies were significantly increased in both groups of patients compared to controls. In encephalopathics acetoacaetate was even more increased than in cirrhotics but ß-hydroxybutyrate and acetate concentrations were decreased. The fact that all ketogenic amino acids are increased as well in cirrhosis would favour a hypothesis of impaired ketone bodies utilisation in the periphery (muscle, brain). The fact that ß-hydroxybutyrate and acetate are significantly decreased in encephalopathic cirrhotics is indicative of an impaired ketogenesis. We observed though that acetoacetate is increased in encephalopathics. Acetoacetate is the main product of ketogenesis and then by using NADH as co-substrate is further metabolised to ß-hydroxybutyrate and acetate in the cellular mitochondria. We can hypothesise that possibly the precarious state of energy production in encephalopathy makes the availability of NADH for this further reaction minimal and it is shifted towards energy production from the Kreb's cycle

which is vital to the hepatocytes instead of finalising a product which is destined for export to other organs like muscle and brain. This is further consolidated by the fact that encephalopathics were shown to have a significantly lower glycerol level which is an indication that fewer triglycerides are broken down and fewer lipids are made available for oxidation which is the main pathway that would lead to ketone body production.

Typical changes in plasma amino acid patterns have been found in different studies in patients (Morgan, 1990; Plauth et al, 1993) and experimental animals in chronic liver failure (Mc Mennamy et al, 1965; Iob et al, 1970). Those changes are increased concentrations of the aromatic amino acids and methionine and decreased concentrations of the branch chain amino acids. This pattern was confirmed in patients in the present study with the exception of isoleucine which was shown to be non significantly increased in cirrhotics. Overall though there was a statistically significant decrease in the concentration of the aromatic amino acids in cirrhotics without encephalopathy.

The aromatic amino acids and methionine are primarily metabolised by the liver and their raised concentrations in both cirrhotics and encephalopathic cirrhotics are probably due to impaired liver metabolism and portosystemic shunting of blood.

The story is more complex for the branch chain amino acids and is further complicated by the findings of this study that in encephalopathics there was a reversal of the well known pattern with high leucine and isoleucine concentrations. The normal liver does not play a major role in the break down of the branch chain amino acids which are mostly broken down in the skeletal muscle and kidneys. It was postulated that hyperinsulinaemia which is present in cirrhosis may drive branch chain

amino acids to the muscle and the kidneys where they are broken down (Tessari et al, 1993). Our results do not support this hypothesis particularly in encephalopathy, as concentrations of leucine and isoleucine are increased in encephalopathy. If we look at branch chain amino acids individually we find that their metabolic fate, after the initial transamination and decarboxylation, can be very different from one to the other. Leucine is a ketogenic amino acid which can be oxidised to acetyl -CoA. This study provides evidence that ketogenesis is impaired in encephalopathy as is also the peripheral utilisation of the ketone bodies. Valine can only be a gluconeogenic amino acid that could enter the Kreb's cycle and provide towards the production of ATP. As acetyl-CoA is in short supply Kreb's cycle can be fuelled from alternative sources as valine. Isoleucine can be metabolised in both ways thus being either gluconeogenic or ketogenic in the body. Isoleucine may as well be feeding the Kreb's cycle in cirrhosis but not as successfully as valine. We do not have an immediate explanation as to why the concentration of isoleucine is high in encephalopathic cirrhotics in our study population. Isoleucine is a ketogenic amino acid and, as the production of acetoacetate is increased but its catabolism is not, it might be an index of diminished ketogenesis in encephalopathy.

Hyperammonaemia and diminished urea production are well characterised phenomena in cirrhotic patients. Our study showed that cirrhotics had decreased levels of glutamine, histidine and arginine but increased levels of glutamate. This is a pattern which is in accordance with a previous study which showed a generalised decrease in those amino acids in chronic liver failure (Plauth et al, 1990). It is in accordance also with studies in experimental animal models of liver failure (De Jong et al, 1995). Although other studies in patients suffering acute liver failure confirmed

a reverse pattern (Fischer et al, 1978), our study in acute liver failure found no differences in any of those substances between patients and controls. Arginine is an amino acid that is an intermediary of the urea cycle. Its observed decreased concentrations are in agreement with a decreased urea cycle as is the decreased histidine concentrations which is a glutamate precursor. Glutamate- glutamine shuttle is an alternative way for ammonia detoxification with increased peripheral glutamine synthesis a pathway for ammonia detoxification. We have also shown that glutamine concentrations in encephalopathics is similar to controls. Although this might seem paradoxical there is evidence of increased ammonia production during encephalopathy, which is implicated in its pathogenesis. The fact that glutamate synthesis is impaired may provide another point for the hyperammonaemia of encephalopathy. An alternative pathway to this is the production of amines and TMAO which can assist in the ammonia detoxification in the presence of urea cycle impairment.

In conclusion this chapter provides evidence that in stable cirrhosis key metabolic pathways are impaired and confirms that there is impaired gluconeogenesis, impaired ketogenesis and ketone bodies break down and impaired urea cycle. In chronic hepatic encephalopathy there the concentrations of the branch chain amino acids are almost normal. Encephalopathics seem to have a unique molecule in their plasma which might be responsible for one of the mechanisms responsible for the pathogenesis of the syndrome. They also have decreased capacity for ammonia detoxification and decreased capacity for fatty acid oxidation. Further studies towards identification of this unique molecule and also towards unification of the different theories on the pathophysiology of hepatic encephalopathy are

guaranteed. It will be important to look at intracellular concentrations of key metabolites in muscle and hepatocytes in patients with cirrhosis in order to measure interorgan exchange of amino acids and its alterations in chronic liver disease and when complications of portal hypertension occur.

REFERENCES

1. Adams RD, Foley JM. Neurological disorder associated with liver disease. Association for Research in Nervous and Mental Disease, Proceedings 1953; 32:198-237
2. Aggarwal S, Kramer D, Yonas H, Obrist W, Kang Y, Martin M, Policare R. Cerebral hemodynamic and metabolic changes in fulminant hepatic failure: A retrospective study. Hepatology 1994;19:80-87
3. Akins R E, Schroedl N A, Gonda S R, Hartzell C H Neonatal rat heart cells cultured in simulated microgravity. In Vitro Cell. Dev. Biol. - Animal 1997;33:337-343
4. Ala Korpela M H-1 NMR Spectroscopy in human blood plasma Prog. NMR Spectrosc. 1995;27:475-497
5. Anand AC, Nightingale P, Neuberger J Early indicators of prognosis in fulminant hepatic failure: an assessment of the King's criteria J Hepatol. 1997; 26: 62-68
6. Anthony PP, Ishak KG, Nayak NC, Poulsen HE, Scheuer PJ, Sobin LH. The morphology of cirrhosis. Recommendation on definition, nomenclature and classification by a working group sponsored by the World Health Organisation Journal of Clinical Pathology 1977 31:395-414
7. Arkadopoulos N, Chen SC, Khalili TM, Detry O, Hewitt WR, Lilja H, et al. Transplantation of hepatocytes for prevention of intracranial hypertension in pigs with ischemic liver failure. Cell Transpl. 1998;7:357-363

8. Asher N, Lake J, Emond J, Roberts J Liver transplantation for fulminant hepatic failure. Arch Surg 1993; 128:677-682

9. Baker T L, Goodwin T J Three - dimensional culture of bovine chondrocytes in rotating wall vessels. In Vitro Cell. Dev. Biol.- Animal 1997;33:358 -365

10. Basile AS, Jones EA, Scolnick P. The pathogenesis and treatment of hepatic encephalopathy: evidence for the involvement of benzodiazepine receptor ligands. Pharmacol. Rev. 1991; 43:27-71

11. Bayly AC, Roberts R A, Dive C Suppression of liver cell apoptosis in vitro by the non-genotoxic hepatocarcinogen and peroxisome proliferator nafenopin. J Cell Biol. 1994;125:197-203

12. Bell JD, Brown JCC, Kubal G, Sadler PJ. NMR invisible lactate in blood-plasma. FEBS Letters 1988;235:81-86

13. Bell JD, Brown JCC, Sadler PJ NMR studies of body fluids. NMR Biomed. 1989;2:246-256

14. Bell JD, Lee JA, Lee HA, Sadler PJ, Wilkie DR, Woodham RH Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: identification of trimethyl-N-oxide. Biochim. Biophys. Acta 1991;1096:101-107

15. Bernal W, Donaldson N, Wyncoll D, Wendon JA. Admission blood Lactate is predictive of outcome in Acetaminophen induced acute liver failure. Hepatology 1999; 30:Suppl.1:420A

16. Bernuau J, Samuel D, Durand F, Bourliere FSM, Adam R, Gugenheim J, et al. Criteria for emergency liver transplantation in patients with acute viral hepatitis and

Factor –V (FV) below 50% of normal - A prospective study. Hepatology 1991;14:Suppl.2: A49

17. Berry MN, Friend DS High yield preparation of isolated rat liver parenchymal cells. J Cell Biol. 1969;43:506-520

18. Bihari D, Gimson AE, Waterson M, Williams R. Tissue hypoxia during fulminant hepatic failure. Crit. Care Med. 1985;13:1034-1039

19. Bismuth H, Samuel D, Castaing D, Williams R, Pereira SP. Liver transplantation in Europe for patients with acute liver failure. Sem Liver Dis 1996; 16:415-425

20. Blei AT. Cerebral edema and intracranial hypertension in acute liver failure: distinct aspects of the same problem. Hepatology 1991; 13:376-379

21. Blei AT, Stolze-Larsen F. Pathophysiology of cerebral edema in fulminant hepatic failure. J Hepatol. 1999;31:771-776

22. Blitzer BL, Waggoner JG, Anthony Jones E, Gralnick HR, Towne D, Butler J et al. A model of fulminant hepatic failure in the rabbit. Gastroenterology 1978; 74:664-671

23. Bloch F, Hansen WW, Packard ME. Nuclear induction Physics Rev 1946;68:127

24. Brodskii VY, Nechaeva NV, Terskikh VV, Novikova TE, Gvazava IG, Fateeva VI. A serum-free medium that preserves normal morphology and a high level of protein synthesis in hepatocytes. Izvestiya Academii Nauk Seriya Biologicheskaya 1996;4: 398-401

25. Butterworth RE, Tracey-Oliver T, Earle L, Lory DJ, White RD, Doolittle DJ et al. Use of primary cultures of human hepatocytes in toxicology studies. Cancer Research 1989;49:1075-1084

26. Canalese J, Gove CD, Gimson AES, Wilkinson SP, Wardle EN, Willams R. Reticuloendothelial system and hepatocyte function in fulminant hepatic failure. Gut 1982; 23: 265-269

27. Chenard-Neu MP, Boudjema K Bernuau J et al. Auxilliary liver transplantation: regeneration of the native liver and outcome in 30 patients with fulminant hepatic failure. A multicentre European study. Hepatology 1996; 23: 1119-1127

28. Chesne C, Guyomard C, Fautrel A, Poullain M-G, Fremond B, De Jong H, et al. Viability and function in primary culture of adult hepatocytes form various animal species and human beings after cryopreservation. Hepatology 1993;18:406-414

29. Chmurne GW, Mellini ML, Halverson D, Issaq HJ, Muschik GM, Urba WJ, McGregor GN et al. A comparison of high performance gel permeation chromatography and Nuclear Magnetic Resonance Spectroscopy in the analysis of plasma from normal subjects and cancer patients. J Lic Chrom 1988;11:647-664

30. Churchill TA, Busza AL, Fuller BJ. Energy metabolism in liver of anoxia tolerant turtle species (Pseudemys scripta): A model for studying hepatic tolerance to cold hypoxia. Cryobiology 1997;35:14-19

31. Clement B, Fremond B, Joly A, Desjardins JF, Campion JP, Guillouzo A. et al. A step towards a bioartificial liver: Extracorporeal supply from isolated hepatocytes. Bull Acad Nation Med 1996;180:1753-1767

32. Clement B, Desille M, Fremond B, Campion JP, Guguen-Guillouzo C, Bourel M, et al. Hepatocytes and cell therapy. Tranf Clin Biol 1998;5:80-87

33. Clemmesen JO, Stolze Larsen F, Kondrup J, Adel Hansen B, Ott P. Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration. Hepatology 1999;29:648-653

34. Coldham NG, Biancotto G, Montesissa C, Howells LC, Sauer MJ. Utility of isolated hepatocytes and radio-HPLC-MSn for the analysis of the metabolic fate of 19-nortestosterone laurate in cattle. Analyst 1998;123: 2589-2594

35. Córdoba J, Gottstein J, Blei AT. Glutamine, *myo*-Inositol, and organic brain osmolytes after portocaval anastomosis in the rat: implications for ammonia-induced brain edema. Hepatology 1996;24:919-923

36. Cytodex :Microcarrier cell culture, principles and methodology . Pharmacia BLK biotechnology 1995.

37. Darr TB, Hubel A. Freezing characteristics of isolated pig and human hepatocytes. Cell Transplantation 1997;6;173-183

38. De Blaaw I, Deutz NEP, Boers W, von Meyenfeldt MF Hepatic amino acid and protein metabolism in non anorectic, moderately cachectic tumour bearing rats. J Hepatol 1997;26:396-408

39. De Jong HC, Deutz NEP, Soeters PB Metabolic adaptation of the kidney to hyperamonnemia during chronic liver insufficiency in the rat. Hepatology 1993;18:890-902

40. De Loecker P, Fuller BJ, Koptelov VA, De Loecker W. Metabolic activity of freshly prepared and cryopreserved hepatocytes in monolayer culture. Cryobiology 1993;30:12-18

41. Demetriou AA, Rozga J Artificial liver support. In: Strain A.J., Diehl A.M., (eds). Liver growth and repair. London: Chapman & Hall, 1998:636-640

42. Demetriou AA Support of the acutely failing liver - State of the art. Annals of Surgery 1998;228:14-15

43. Denton RM, Halestrap AP Regulation of pyruvate metabolism in mammalian tissues. Essays Biochem 1978;15:37-44

44. Diaz-Buxo J, Blumenthal S, Hayes D, Gores P, Gordon B. Galactosamine-induced fulminant hepatic necrosis in unanesthetized canines. Hepatology 1997; 25:950-957

45. Dickinson WC. Dependence of the F^{19} nuclear resonance position on chemical compound. Phys. Rev 1950;77:736-737

46. Diener B, Utesch D, Beer N, Durk H, Oesch F A method for the cryopreservation of liver parenchymal cells for studies of xenobiotics. Cryobiology 1993;30:116-127

47. Donato MT, Castell JV, Gomez-Lechon MJ Characterization of drug metabolising activities in pig hepatocytes for use in bioartificial liver devices: comparison with other hepatic cellular models. J Hepatol 1999;31:542-549

48. Duee PH, Pegorier JP, Quant PA, Herbin C ,Kohl C, Girard J Hepatic ketogenesis in new born pigs is limited by low mitochondrial 3-hydroxy-3-methylglytaryl-Coa synthase activity. Biochem J 1994:;298;207-212

49. Du Ruisseau JP, Greenstein JP, Winitz M, Birnbaum SM Studies on the metabolism of amino acids and related compounds *in vivo.* IV. Blood ammonia and urea levels following intraperitoneal administration of amino acids and ammonium acetate and the effect of arginine thereon. Arch Biochem Biophys 1956;64:355-370

50. Engan T, Crane J, Kvinnsland S. Proton Nuclear Magnetic Resonance Spectroscopy of methylene and methyl line widths in plasma- significant variation with extent of breast cancer duration of pregnancy and ageing. NMR Biomed. 1991;4:142-149

51. Ernst RR Anderson WA Application of Fourier Transform Spectroscopy to magnetic resonance. Rev. Sci. Instrum. 1966;37:93-102

52. Eugene M, de Certaines J, Le Moyec L, Le Rumeur E, Desruennes M, Lechat P, Cabrol C Proton Magnetic Resonance Spectroscopy of plasma during heart transplantation C.R. Acad. Sci. Paris 1988;307(III): 41-45

53. Eugene M, Le Moyec L, Descertaines J, in Magnetic Resonance Spectroscopy of Biofluids: a new tool in clinical biology. World Scientific Editors 1989, p.179-186

54. Eugene M, Le Moyec L, de Certaines J, Desruennes M, Le Rumeur E, Fraysse JB, Cabrol C Lipoproteins in heart transplantation: Proton Magnetic Resonance Spectroscopy of plasma. Magn Reson. Med. 1991;18:93-101

55. Fahey JL Toxicity and blood ammonia rise resulting from intravenous amino acid administration in man: the protective effect of L-Arginine. JCI 1957:1647-1655

56. Fan TWM. Metabolic profiling by one and two dimensional NMR analysis of complex mixtures. Prog. NMR Spectrosc. 1996;28:161-189

57. Fischer JE, Baldessarini RJ. Pathogenesis and therapy of hepatic coma. In: Popper H, Schaffner F (eds). Progress in liver diseases (vol. 5). Grune and Stratton New York 1976; p.363-397

58. Fischer JE, Rosen HM, Ebeid AM, James JH, Keane JM, Soeters PB The effect of normalization of plasma amino acids on hepatic encephalopathy in man. Surgery 1978;80:77-91

59. Flendrig LM, La Soe JM, Jorning JJA, Steenbeek A, Karlsen OT, Bovee WMMJ, et al. In vitro evaluation of a novel bioreactor based on an integral oxygenator and a spirally wound non woven polyester matrix for hepatocyte culture as small aggregates. J Hepatol1997;26:1379-1392

60. Flendrig LM, Chamuleau RAFM, Maas MAW, Daalhuisen J, Hasset B, Kilty CG, et al. Evaluation of a novel bioartificial liver in rats with complete liver ischaemia : treatment efficacy and species - specific alpha-GST detection to monitor hepatocyte viability. J Hepatol 1999;30:311-320

61. Fossel ET, Carr JM , McDonagh J Detection of malignant tumours. Water suppressed proton nuclear magnetic resonance spectroscopy of human plasma. NEJM 1986;315:1369-76

62. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, et al. Treatment of Crigler-Najjar syndrome type I with hepatocyte transplantation. NEJM 1998;338:1422-1426

63. Foxall PJD, Price RG, Jones JK, Neild GH, Thompson FD, Nicholson JK High resolution proton magnetic resonance spectroscopy of cyst fluids from patients with polycystic kidney disease. Biochem. Biophys. Acta 1992;1138:305-314

64. Foxall PJD, Parkinson JA, Sadler IH, Lindon JC, Nicholson JK Analysis of Biological fluids using 600 MHz Proton NMR Spectroscopy: Application of homonuclear 2-dimentional J-resolved spectroscopy to urine and blood-plasma for spectral simplification and assignment. J Pharmac Biomed Anal 1993;11:21-31

65. Foxall PJD, Mellotte JG, Bending MR, Lindon JC, Nicholson JK. NMR Spectroscopy as a novel approach to the monitoring of renal transplant function. Kidney International 1993;43,:234-245

66. Foxall PJD, Spraul M, Farrant RD, Lindon JC, Neild GH, Nicholson JK 750 MHz 1H-NMR Spectroscopy of Human blood-plasma J Pharm Biomed Anal 1993;11:267-276

67. Foxall PJD, PhD thesis, University of London, 1993

68. Francavilla A, Makowka L, Polimeno L, Barone M, Demetris J, Prelich J, Van Thiel DH, Starzl TE. A dog model for acetaminophen induced fulminant hepatic failure. Gastroenterology 1989;96:470-478

69. Friedman SL. The cellular basis of hepatic fibrosis. The mechanisms and treatment strategies. NEJM. 1993;328:1828-1835

70. Fuchs M, Gerlach J, Unger J, Encke J, Smith M, Neuhaus P, at al. ?-ketoacid metabolism by hepatocytes cultured in a hybrid liver support bioreactor. Int J Artif Organs 1994;17:554-558

71. Fuller BJ, Busza AL. Proton NMR studies on the permeation of tissue fragments by dimethyl sulfoxide-liver as a model for compact tissues. Cryobiology 1994;15:131-134

72. Gartland KPR, PhD Thesis, University of London 1988

73. Gerlach J, Kloppel K, Schauwecker HH, Tauber R, Muller C, Bucherl ES. Use of hepatocytes in adhesion and suspension cultures for liver support bioreactor. Int J Artific Organs 1989; 12:785-791

74. Gerlach JC, Brombacher J, Kloppel K, Schnoy N, Neuhaus P Comparison of four methods of mass hepatocyte isolation from pig and human livers. Transplantation 1994; 57:1318-1322

75. Gerlach J, Zeimer R, Neuhaus P Fulminant liver failure: relevance of extracorporeal hybrid liver support systems. Int J Artif Org 1996;19:7-13

76. Gerlach JC, Fuchs M, Smith MD, Bornemann R, Encke J, Neuhaus P, Riedel E Is a clinical application of hybrid liver support systems limited by an initial disorder in cellular aminoacid and α-keto acid metabolism rather than by later gradual loss of primary hepatocyte function? Transplantation 1996;62:224-228

77. Goldstein GW The role of brain capillaries in the pathogenesis of hepatic encephalopathy. Hepatology 1984; 4:565-567

78. Goodwin T J, Prewett TL, Wolf D A, Spaulding GF Reduced shear stress: a major component in the ability of the mammalian tissue to form three - dimensional assemblies in simulated microgravity. J Cell Biochem 1993; 51: 310-311

79. Goodwin TJ, Prewett TL, Spaulding G F, Becker JL Three dimensional culture of a mixed mullerian tumor of the ovary: expression of *in vivo* characteristics. In Vitro Cell. Dev. Biol. - Animal 1997:33;366 -374

80. Gosden RG, Sadler IH, Reed D, Hunter RHF Characterisation of ovarian follicular fluids of sheep, pigs and cows by using proton nuclear magnetic resonance spectroscopy. Experientia 1990; 46: 1012-1015

81. Grasdalen H., Belton PS, Pryor JS., Rich GT. Quantitative proton magnetic resonance from plasma from uremic patients during dialysis. Mag Res Chem. 1987;225:811 - 816

82. Guillouzo A, Morel F, Langouet S, Maheo K, Rissel M Use of hepatocyte cultures for the study of hepatotoxic compounds. J Hepatol 1997;26; Suppl 2:73-80

83. Guzic K, Reiss K, Korohoda W Survival and Phenol red metabolism in chick-embryo hepatocytes cultured in the serum-free Medium 199 supplemented with

Dextran T-500 and antioxidants. Folia Histochemica et Cytobiologica 1989; 27: 125-127

84. Harrison PM, Keays R, Bray GP, Alexander GJM, Williams R. Improved outcome of paracetamol-induced fulminant hepatic failure by late administration of acetylcysteine. Lancet 1990; 335:1572-1573

85. Harrison PM, O'Grady JG, Keays RT, Alexander GJM Williams R. Serial prothrombin time as prognostic indicator of acetaminophen-induced fulminant hepatic failure. BMJ 1990; 301:964-966

86. Hayes PC, Portal Hypertension revisited. Quarterly J Med. 1995;88:751-754

87. Herring FG, Phillips PS, Pritchard PH. Proton Magnetic Resonance Spectroscopy of plasma from patients with dyslipidoproteinemia- identification of factors governing methyl and methylene proton line widths. J Lipid Res 1989;30:521-528

88. Hewage CM, PhD thesis, University of Edinburgh, 1995

89. Höfeler H, Scheulen ME Monitoring of patients with non-seminomatous testicular cancer by Nuclear Magnetic Resonance spectroscopy of plasma. Eur J Cancer Clin Oncol 1989;25:1141-1144

90. Hoofnagle J, Carithers R Jr, Shapiro C, Asher N Fulminant hepatic failure; Summary of a Workshop. Hepatology 1995; 21:240-252

91. Hoult DI Solvent peak saturation with single phase and quadrature Fourier transformation. J Magn Reson 1976; 21:337-340

92. Ijima H, Matushita T, Nakazawa K, Fujii Y, Funatsu K Hepatocyte spheroids in polyurethane foam: Functional analysis and application for a hybrid artificial liver. Tissue Engineering 1998:4:213-226

93. Iles RE, Hind AJ, Chalmers RA Use of proton nuclear magnetic resonance spectroscopy in detection and study of aciduria. Clin Chem 1985;31:1795-1801

94. Ingram M, Techy GB, Saroufeem R, Yazan O, Narayan KS, Goodwin TJ, Spalding GF Three dimensional growth patterns of various human tumor cell lines in simulated mirogravity of a NASA bioreactor. In Vitro Cell. Dev. Biol. - Animal 1997;33:459 - 466

95. Iob V, Mattson WJ Jr, Sloan M, Coon WW, Turcotte JG, Child CG III Alteration in plasma free amino acids in dogs with hepatic insufficiency. Surg Gynecol Obstet 1970;130:794-800

96. Jalan R, Chung YL, Redhead DN, Hayes PC, Bell JD, Steiner RE *In vitro* Proton (^{1}H) Magnetic Resonance Spectroscopy of the plasma in cirrhosis and changes following the transjugular intrahepatic portosystemic stent shunt (TIPSS). Hepatology 1995;22;Pt2SS:1310

97. Jalan R, Seery JP, Taylor-Robinson SD. Review article: Pathogenesis and treatment of chronic hepatic encephalopathy. Alim Pharm Ther 1996;10:681-697

98. Jalan R, Damink SWMO, Deutz NEP, Lee A, Hayes PC. Moderate hypothermia for uncontrolled intracranial hypertension in acute liver failure. Lancet 1999; 354: 1164-1168

99. Janke J, Gerlach J, Kardassis D, Bohmer C, Rossaint R Effect of a hybrid liver support system on cardiopulmonary function on healthy pigs. Int J Artif Org 1997;20:570-575

100. Jauregui HO, Naik S, Santangini H, Trenkler DM, Mullon CJP The use of microcarrier roller bottle culture for large scale production of porcine hepatocytes. Tissue Engin 1997;3:17-25

101. Jeener J. Ampère Summer School Basco Polje Yugoslavia 1971

102. Jessup JM, Brown D, Fitzgerald W, Ford RD, Nachman A, Goodwin TJ, Spaulding G Induction of carcinoembryogenic antigen expression in a three dimensional culture system In Vitro Cell. Dev. Biol.- Animal 1997;33:352-357

103. Jones EA, Gammal SH, Martin P Hepatic encephalopathy: new light on an old problem. Quarterly J Med. 1988; 69:851-867

104. Jungermann K, Katz N Functional hepatocellular heterogeneity. Hepatology 1982;2:385-395

105. Kalpana K, Ong HS, Soo KC, Tan SY, Prema Raj J An improved model of Galactosamine-induced fulminant hepatic failure in the pig. Journal of Surgical Research 1999; 82:121-130

106. Kasai S, Mito M Large scale cryopreservation of isolated dog hepatocytes. Cryobiology 1993;30:1-11

107. Kato M, Hughes RD, Keays RT, Williams R Electromicroscopic study of brain capillaries in cerebral edema from fulminant hepatic failure. Hepatology 1992; 15:1060-1066

108. Khaoustov V L, Darlington GJ, Soriano HE, Krishnan B, Risin D, Pellis NR, Yoffe B Induction of three dimensional assembly of human liver cells by simulated microgravity. In vitro Cell. Dev. Bio.- Animal 1999;35:501-509

109. Kinsell LW, Harper HA, Barton HC, Hutchin ME, Hess JR Studies in methionine and sulfur metabolism. The fate of iv. administered methionine in normal individuals and patients with liver damage. JCI 1948;27:677- 688

110. Kloth S, Edenbeck C, Kubitza M Stimulation of renal organotypic development under organotypic vascular conditions. FASEB J. 1995;9:963-967.

111. Koebe H G, Dahnhardt C, Muller-Hocker J, Wagner H, Schildberg J Cryopreservation of porcine hepatocyte cultures. Cryobiology 1996;33:127-141

112. Koebe HG, Schildberg FW Isolation of porcine hepatocytes from slaughterhouse organs. Int J Artif Org 1996 ;19:53-60

113. Koukoulis G, Rayner A, Tan K-C, Williams R, Portmann B. Immunolocalization of regenerating cells after submassive liver necroses using PCNA staining. Journal of Pathology 1992; 166:359-368

114. Kriat M, Confort-Gouny S, Vion-Dury J, Viout P, Cozzone PJ. 2 dimensional H-1-NMR Spectroscopy of normal and pathological human plasma. Complete water suppression and further assignment of resonances. Biochimie 1992;74:913-918

115. Kriat M, Confort-Gouny S, Vion-Dury J, Sciaky M, Viout P, Cozzone PJ Quantitation of metabolites in human blood serum by Proton Magnetic Resonance Spectroscopy. A comparative study of the use of formate and TSP as concentration standards. NMR Biomed. 1992; 5:179-184

116. Krieger D, Krieger S, Jansen O, Gass P, Theilmann L, Lichtnecker H. Manganese and chronic hepatic encephalopathy. Lancet 1995; 346: 270-274

117. Lang JM, Wilmore DW Is glutamine a conditionally essential amino acid ? Nutr Rev 1990; 48:297 -307

118. Lawrence JN, Bedford DJ Development of an optimal method for the cryopreservation of hepatocytes and their subsequent monolayer culture. Toxic in vitro 1991;5:39-50

119. Lee W Acute liver failure. NEJM. 1993;329:1862-72

120. Lelkes PI, Galvan D I, Hayman G T, Goodwin T J, Chatmen D Y, Cherian S, Garcia RMG, et al. Simulated microgravity conditions enhance differentiation of

cultured P12 cells towards the neuroendocrine phenotype. In Vitro Cell. Dev. Biol. - Animal 1998;34:416-325.

121. Leweling H, Kortsik C, Gladisch R, Staedt U, Hagmuller E, Holm E Effects of ornithine-aspartate on plasma ammonia and plasma amino acids in patients with liver cirrhosis. A double blind, randomised study using a four fold crossover design. In: Bengtsson F, Jeppsson B, Almdal T, Vilstrup H, (eds). Progress in hepatic encephalopathy and metabolic nitrogen exchange. Boca Raton, Fla: CRS Press 1991;377-389

122. Lindon JC, Nicholson JK, Everet JR NMR Spectroscopy of biofluids. Annual reports on NMR Spectroscopy 1999;38:1-87

123. Liu M, Nicholson JK, Parkinson JA, Lindon JC Measurement of biomolecular diffusion coefficients in blood-plasma using two dimensional H-1-H-1 diffusion-edited total correlation NMR Spectroscopy. Anal Chem 1997;69:1504-1509

124. Liu M, Mao X, Ye C, Huang H, Nicholson JK, Lindon JC Improved watergate pulse sequence for solvent suppression in NMR Spectroscopy J Magn Reson 1998;132:125-131

125. Lockett CJ, Busza AL, Proctor E, Churchill TA, Williams SR, Fuler BJ Proton Nuclear Magnetic Resonance Spectroscopy of lactate production in isolated rat liver during cold preservation. Cryobiology 1996;33:271-275

126. Lockwood AH Hepatic encephalopathy: experimental approaches to human metabolic encephalopathy. CRC Critical reviews in Neurobiology 1987; 3:105-133

127. Lockwood AH, Yap EWH, Wong W-H Cerebral ammonia metabolism in patients with severe liver disease and minimal hepatic encephalopathy. J Cerebr Blood Flow Metab 1992; 11:337-341

128. Long JP, Pierson S, Hughes JH Rhinovirus replication in HeLa cells cultured under conditions of simulated microgravity. Aviation, Space and Environmental Medicine 1998;69:851-856

129. Long J P, Pierson S, Hughes J H. Suppression of Epstein - Barr virus reactivation in lymphoblastoid cells cultured in simulated microgravity. In Vitro Cell. Dev. Biol. - Animal 1999;35: 49-54

130. Maganto P, Cienfuegos A, Santamaria L, Eroles G, Andres S, Castillo - Olivares JL, et al. Cryopreservation and transplantation of hepatocytes: An approach for culture and clinical application. Cryobiology 1988;25:311-322

131. Marsh DC, Hjelmhaug JA, Vreugdenhil PK, Kerr JA, Rice M J, Belzer FO et al. Hypothermic preservation of hepatocytes. III. Effects of resuspension media on viability after up to 7 days of storage. Hepatology 1991;13:500-508

132. Matkowskyj KA, Marrero JA, Carroll RE, Danilkovich AV, Green RM, Benya RV. Azoxymethane-induced fulminant hepatic failure in C57BL/6J mice: characterization of a new animal model. Am J Physiol 1999: 277:G455-G462

133. McCashland TM, Shaw BW, Tape E The American experience with transplantation for acute liver failure. Semin Liver Dis 1996; 16:427-433

134. McCullough AJ, Galmour T Differences in amino acid kinetics in cirrhosis. Gastroenterology 1993;104:1858-1864

135. McMenamy RH, Vang JH, Drapanas T Amino acid and alpha-keto acid concentrations in plasma and blood of the liverless dog. Am J Physiol 1965;209:1046-1052

136. Michalopoulos GK Liver regeneration: Molecular mechanisms in growth control. FASEB 1990;4:176-187

137. Mims MP, Morrisett JD, Mattioli CA., Gotto AM Effect of triglyceride levels in methyl and methylene envelope line widths in Proton Nuclear Magnetic resonance Spectroscopy of human plasma. NEJM 1989;320:1452-1457

138. Morgan MY, Milsom JP, Sherlock S Plasma ratio of valine, leucine and isoleucineto phenylalanine and tyrosine in liver disease. Gut 1978;19:1068-1073

139. Morgan MY, Marshall AW, Milsom JP, Sherloch S Plasma amino acid patterns in liver disease. Gut 1982;23:362 -370

140. Morgan MY Branched chain amino acids in the management of chronic liver disease. Facts and fantasies. J Hepatol 1990; 11:133-141

141. Morrison DK, Chapes SK, Guikema JA Experiments with suspended cells on the space shuttle. Physiologist 1992;35:S31-S34

142. Mountford CE, May GL, Wright LC, Mackinnon WB, Dyne M, Holmes KT, van Haaften-Day C, Tattersall MHN Protolipid identified by Magnetic Resonance Spectroscopy in plasma of a patient with borderline ovarian tumour. Lancet 1987;8537:829-832

143. Nagayama K, Kumar A , Wüthrich K, Ernst RR Experimental techniques of Two - Dimensional Correlation Spectroscopy. J Magn Reson 1980;40:321-324

144. Naik S, Trenkler T, Santangini H, Mullon CJP, Solomon BA, Pan J, Jauregui HO Functional recovery of porcine hepatocytes after hypothermic or cryogenic preservation for liver support systems. Cell Transplantation 1997;6:447-454

145. Nakamura J, Okamoto T, Schumacher IK, Tabei I, Chowdhury NR, Chowdhury JR, et al. Treatment of surgically induced acute liver failure by transplantation of conditionally immortalised hepatocytes. Transplantation 1997;63:1541-1547

146. Nelson LJ, Newosome PN, Howie AF, Hadoke PWF, Dabos KJ, Walker SW, Hayes PC et al. An improved ex-vivo method of primary porcine hepatocyte isolation for use in bioartificial liver systems. Europ J Gastro Hepatol 2000;12:923-930

147. Newsome PN, Plevris JP, Nelson LJ, Hayes PC Animal models of fulminant hepatic failure. Liv Transp Surg 2000;6:21-31

148. Nicholson JK, Buckingham MJ, Sadler PJ High resolution H-1 NMR studies of vertebrate blood and plasma. Biochem J 1983;211:605-615

149. Nicholson JK, Timbrell JA Sadler PJ Proton NMR spectra of urine as indicators of renal damage.- Mercury induced nephrotoxicity in rats. Mol Pharmacol 1985;27: 644-651

150. Nicholson JK, Wilson ID Higher Resolution Proton Magnetic Resonance Spectroscopy of biological Fluids. Prog NMR Spectros 1989;8:449-501

151. Nicholson JK, Foxall PJD, Spraul M, Farrant RD, Lindon JC 750-MHz H1 and H1-C13 NMR Spectroscopy of human blood-plasma Anal Chem 1995;67:793-811

152. Nicholson JK, Foxall PJD, Spraul M, Farrant RD, Lindon JC 750 MHz ^{1}H and ^{1}H-^{13}C NMR Spectroscopy of human blood plasma. Anal Chem 1995;67:793-811

153. Nishina M, Hori E, Matsushita K, Takahashi M, Ohsaka A. 1H Nuclear Magnetic Resonance Spectroscopy of serums from patients with malaria. Physiol. Chem. Phys. Med. NMR 1988;20:269-271

154. Norenberg MD, Mozes LW, Papendick RE, Norenberg LOB Effect of ammonia on glutamate, GABA and rubidium uptake by astrocytes. Ann. Neurol. 1985; 18:149-155

155. Oesch F, Abdel-Latif H, Diener B Xenobiotic metabolising enzyme activities and viability are well preserved in EDTA isolated rat liver parenchymal cells after cryopreservation. In Vitro Cell Devel Biol.-Animal 1995;31:590-599

156. O'Grady JG, Gimson AES, O'Brien CJ, Pucknell A, Hughes RD, Williams R. Controlled trials of charcoal hemoperfusion and prognostic factors in fulminant hepatic failure. Gastroenterology 1988; 94:1186-1192

157. O'Grady J, Alexander GJM, Hayllar KM, Williams R. Early indicators of prognosis in fulminant hepatic failure. Gastroenterology 1989; 97:439-445

158. O'Grady JC, Schalm SW, Williams R. Acute liver failure: redefining the syndromes. Lancet 1993; 342:273-275

159. Ohsaka A, Yoshikawa K, Matuhasi T Detection by Proton Nuclear Magnetic Resonance of elevated lactate concentration in serums from patients with malignant tumors. Jpn. J. Med. Sci. Biol. 1979;32:305-313

160. Olasmaa M, Rothstein JD, Guidotti A. Endogenous benzodiazepine receptor ligands in human and animal hepatic encephalopathy. J Neurochem 1990; 55:2015-2023

161. Ono J, Hutson DJ, Dombro RS, Levi JU, Livingstone A, Zeppa R. Tryptophan and hepatic coma. Gastroenterology 1978 74:196-200

162. Otvos JD, Coffer MC, Chen S, Wehrli S. An improved method for the detection of malignancy by Proton NMR Spectroscopy of plasma. Biochem Biophys Res Commun 1987;145:1397-1405

163. Paine AJ, Hockin LJ The maintenance of cytochrome *P*-450 in liver cell culture: Recent studies on *P*-450 mediated mechanisms of toxicity. Toxicology 1982;25:41-45

164. Pauwels A, Mostefa-Kara N, Florent C, Lévy VG Emergency liver transplantation for acute liver failure; evaluation of London and Clichy criteria. J Hepatol 1993; 17:124-127

165. Peeling J, Shoemaker L, Gauthier T, Benarroch A, Sutherland GR, Minuk GY. Cerebral metabolic and histological effects of thioacetamide-induced liver failure. Am J Physiol 1993; 265:G573-G578

166. Percy-Robb IW, Finlayson NDCF. Clinical chemistry of liver diseases. In: Diseases of the gastrointestinal tract and liver (eds) Shearman DJC. , Finalyson NDCF., Camillieri M., Carter DC. Churchill Livingstone 1997;735-762

167. Petersen KF, Cline GW, Blair JB, Shulman JI Substrate cycling between pyruvate and oxaloacetate in awake normal and 3,3'-5-triiodo-L-thyronine treated rats. Am J Physiol 1994;267:E273-E277

168. Petersen KF, Blair JB, Shulman JI Triiodothyronine treatment increases substrate cycling between pyruvate carboxylase and malic enzyme in perfused rat liver. Metabolism Clinical and Experimental 1995;44:1380-1383

169. Platt KL, Charisse H, Tommasone M, Diener B, Oesch F. Direct analysis of phase I metabolites, phenol sulphates, glucuronides and glutathione conjugates of

Benzo[*A*]pyrenin freshly isolated, hypothermically stored and cryopreserved hepatocytes. Polycyclic Aromatic Compounds 1996;10:67-75

170. Plauth M, Egberts EH, Abele R, Muller PH, Furst P Characteristic pattern of free amino acids in plasma ands skeletal muscle in stable hepatic cirrhosis. Hepato-gastroenterology 1990;37:135-139

171. Plauth M, Egberts EH, Hamster W, Torok M, Muller PH, Furst P et al. Long term treatment of latent portosystemic encephalopathy with branch chain amino acids. A double-blind placebo-controlled crossover study. J Hepatol 1993;17:308-314

172. Plevris JN, Schina M, Hayes PC Review article: the management of acute liver failure Alim Pharmacol Ther 1998; 12:405-418

173. Pomier-Layrargues GP, Giguere JF, Lavoie J et al. Flumazenil in cirrhotic patients in hepatic coma: a randomised double blind placebo-controlled crossover trial. Hepatology 1994; 19:32-37

174. Pomier-Layrargues G, Shapcott D, Spahr L, Butterworth RF Accumulation of manganese and copper in the pallidum of cirrhotic patients: role in the pathogenesis of hepatic encephalopathy? Metab. Brain Dis. 1995; 10:351-354

175. Pont H, Vion-Dury J, Criat M, Mouly-Bandini A, Sciaky M, Viout P, Confort-Gouny S, et al. NMR Spectroscopy of plasma during acute rejection of transplanted hearts. Lancet 1991;337:792-793

176. Portmann B, Talbot IC, Day DW, Davidson AR, Murray-Lyon IM, Williams R. Histopathological changes in the liver following a paracetamol overdose: correlation with clinical and biochemical parameters. Journal of Pathology 1975; 117:169-181

177. Poullain M-G, Fautrel A, Guyomard C, Chesne C, Grislain L, Guillouzo A Viability and primary culture of rat hepatocytes after hypothermic preservation: The superiority of the Leibovitz medium over the University of Wisconsin solution for cold storage. Hepatology 1992;15:97-106

178. Pow EM, Schols AMWJ, Deutz NEP, Wouters EFM Plasma and muscle amino acid levels in relation to resting energy expenditure and inflammation in stable chronic obstructive pulmonary disease Am J Resp Crit Care Med 1998;158:797-801

179. Purcell EM., Torrey HC, Pound RV Resonance absorption by nuclear magnetic moments in a solid. Physics Rev.1946:68:37-38

180. Raabe W Synaptic transmission in ammonia intoxication. Neurochem Pathol 1987; 6:145-166

181. Rappaport AM. The structural and functional unit in the human liver (liver acinus) Anat. Rec.1958; 130: 673-687

182. Ratanasavanh D, Baffet G, Latinier MF, Rissel M, Guillouzo A Use of hepatocyte co-cultures in the assessment of drug toxicity from chronic exposure. Xenobiotica 1988;18:765-771

183. Record C, Buxton B, Chase R, et al. Plasma and brain amino acids in fulminant hepatic failure and their relationship to hepatic encephalopathy. Eur J Clin Invest. 1976; 6:387-394

184. Ringe B, Lubbe N, Kuse E, Frei U, Pichlmayr R Total hepatectomy and liver transplantation as two stage procedure. Ann Surg 1993; 218:3-9

185. Sakai Y, Naruse K, Nagashima I, Muto T, Suzuki M Short-term hypothermic preservation of porcine hepatocyte spheroids using UW solution. Cell Transplantation 1996;5:505-511

186. Samuel D Artificial liver support systems. Biodrugs 1997:8;S1;26-28

187. Saunders JB, Walters JRF, Davies P, Paton A. A 20-year prospective study of cirrhosis. British Medical Journal 1981; 282:263-266

188. Schiodt FV, Atillasoy E, Shakil AO, Caldwell C, Kowdley KV, Stribling R, et al. Etiology and outcome for 295 patients with acute liver failure in the United States. Liver Transplantation and Surgery 1999;5:29-34

189. Schumacher JH, Conrad D, Manke HG, Clorius JH, Matys ER, Hauser H, et al. Investigations concerning the potential for using H-1 NMR Relaxometry or high resolution spectroscopy of plasma as a screening test for malignant lung disease Magn Reson Med 1990;13:103-132

190. Scott CD, Baxter RC Synthesis of the acid-labile subunit of the growth-hormone-dependent Insulin-like-growth-factor-binding protein complex by rat hepatocytes in culture. Biochem J 1991; 275: 441-446

191. Seglen PO Autoregulation of glucolysis, respiration, gluconeogenesis and glycogen synthesis in isolated parenchymal rat liver cells under aerobic and anaerobic conditions. Biochim Biophys Acta 1974;338:317-336

192. Seifter S, Englard S Energy metabolism. In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA (eds). The liver: Biology and pathobiology, New York: Raven Press,1994:323-364

193. Sheil AGR, Sun J, Mears DC, Waring M, Woodman K, Johnston B, et al. Positive biochemical effects of a bioartificial liver support system (BALSS) in a porcine model of fulminant hepatic failure (FHF). Int J Artif Org 1998;21:43-48

194. Shiraha H, Koide N, Hada H, Ujike K, Nakamura M, Shinji T, et al. Improvement of serum amino-acid profile in hepatic failure with the bioartificial liver using multicellular hepatocyte spheroids. Biotechn Bioengin 1996;50: 416-421

195. Sielaff TD, Hu MY, Rao S, Groehler K, Olson D, Mann HJ, et al. A technique for porcine hepatocyte harvest and description of differentiated function in static culture. Transplantation 1995; 59:1459-1463.

196. Sielaff TD, Hu MY, Rollins MD, Bloomer JR, Amiot B, Hu WS, Cerra FB. An anesthetized model of lethal canine galactosamine fulminant hepatic failure Hepatology 1995; 21:796-804

197. So PW, Busza AL, Fuler BJ Metabolic effects of citrate in liver during cold hypoxia studied by H-1 NMR spectroscopy. Cryobiology 1998;36:225-235

198. Smith ICP, Smart CR, Feller W, Bunnag W, Miya F, Hughes P, et al. A clinical trial on the use of magnetic resonance spectroscopy for the detection of cancer. The Cancer Journal 1991;4:125-130

199. Strauss G, Hansen BA, Kirkegaard P, Rasmussen A, Hjortrup, Stolze Larsen F. Liver function, cerebral blood flow autoregulation and hepatic encephalopathy in fulminant hepatic failure. Hepatology 1997;25:837-839

200. Strom SC, Jirtle RL, Michalopoulos GA Genotoxic effects of 2-acetylaminofluorene on rat and human hepatocytes. Environ Health Perspect 1983;49:165-170

201. Sun E L, Aspar DG, Ulrich RG, Melchior GW Cryopreservation of cynomologus monkey (*Macaca fascicularis*) for subsequent culture and protein synthesis studies. In Vitro Cell. Devel. Biol. 1990;25:147-150

202. Sun R, Gimbel HV, Liu S, Guo DL, Hollenberg MD Effect of diclofenac sodium and dexamethasone on cultured human Tenon's capsule fibroblasts. Opth Res Las 1999;30: 382-388

203. Sussman NL, Chong MG, Koussayer T, He D, Shang TA, Whisennand HH Reversal of fulminant hepatic failure using an extracorporeal liver assist device. Hepatology 1992;16:60-65

204. Sze DY, Jardetzky O High Resolution Proton NMR Studies of Lymphocyte Extracts. Immunomethods 1994;4:113-126

205. Takahashi Y, Kumada H, Shimuzu M, Tanikawa K, Kumashiro R, Omata M, Ehata T, et al. A multicentre study on the prognosis of fulminant viral hepatitis: early prediction for liver transplantation. Hepatology 1994; 19: 1065-1071

206. te Velde AA, Ladiges NCJJ, Flendrig LM, Chamuleau RAFM Functional activity of isolated pig hepatocytes attached to different extracellular matrix substrates: implications for application of pig hepatocytes in a bioartificial liver. J Hepatol 1995;23:184-192

207. Tessari P, Biolo G, Inchiostro S, Orlando R, Vettore M, Sergi G Leucine and phenylalanine kinetics in compensated liver cirrhosis: effects of insulin. Gastroenterology 1993;104:1712-1721

208. Thomson MB, Davis DG, Morris RW Taurine conjugate of 3-Alpha, 6-Beta, 7-Beta-trihydroxy-5-Beta,22-Cholen-24-oic acid (Tauro-Delta-22-Beta-Muricholate) - The major Bile-acid in the serum of female rats treated with alpha

naphthylisothiocyanate and its secretion in liver slices. J Lipid Research 1993;34:553-561

209. Tibbs C, Williams R Viral causes and management of acute liver failure. J Hepatol 1995; 22 (suppl.1):68-73

210. Toshchakov VY, Morozova GI, Onishchenko NA Energy metabolism of cryopreserved rat hepatocytes. Cryo-letters1991;12:259-272

211. Trey C, Davidson LS. The management of fulminant hepatic failure. In: Popper H, Schaffner F, (eds). Progress in liver disease. New York: Grune&Stratton,1970: 282-298

212. Vara E, Arias-Diaz H, Villa N, Hernandez J, Cruz G, Ortiz P, et al. Beneficial effect of S-adenosylmethionine during both cold storage and cryopreservation of isolated hepatocytes. Cryobiology 1995;32: 422-427

213. Villeneuve J-P, Dagenais M., Huet P-M., Roy A, Lapointe R, Marleau D. The hepatic microcirculation in the isolated perfused human liver. Hepatology 1996 23:24-31

214. Voorhies TM, Ehrlich ME, Duffy TE, Petito CK, Plum F Acute hyperammonemia in the young primate: physiologic and neuropathologic correlates. Pediatric Research 1983; 17:970-975

215. Wang X, Andersson R Hepatocyte transplantation: A potential treatment for acute liver failure. Scand J Gastroenterol1995; 30:193-200

216. Watanabe FD, Mullon CJP, Hewitt WR, et al. Clinical experience with a bioartificial liver in the treatment of severe liver failure. Ann Surg 1997; 225:484-491

217. Watts P, Smith MD, Edwards I, Zammit V, Brown V, Grant H. The influence of medium composition on the maintenance of cytochrome P-450, glutathione content and urea synthesis: a comparison of rat and sheep primary hepatocyte cultures J. Hepatology 1995; 23:605-612

218. Watts P, Grant MH Cryopreservation of rat hepatocyte monolayer cultures. Human and Experim. Toxic 1996;15:30-37

219. Wei Y, Detre K, Everhart J The NIDDK Liver Transplantation Database. Liver Transplant Surg 1997; 3:10-22

220. Wendon JA, Harrison PM, Keays R, Williams R Cerebral blood flow and metabolism in fulminant liver failure. Hepatology 1994;19:1407-1413

221. Wilkinson SP, Weston MJ, Parsons V, Williams R Dialysis in the treatment of renal failure in patients with liver disease. Clinical Nephrology 1977; 8:287-292

222. Wilkinson SP, Blendis LM, Williams R Frequency and type of renal and electrolyte disorders in fulminant hepatic failure. British Medical Journal 1974; 1:186-189